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# FASTA Search Result

The Reference 1

Computed at GenomeNet FASTA Server (Kyoto Center) on Wed Jun 2 20:04:54 JST 2004

Database Name NR-AA

>query

1M1EINDLKK SFGVRILWQG LSHKFLPGTM TALTGASGSG KSTLLNCLGT  
L51DKPSSGQ ILVEDVDLLK LSTRKQRLYR KNTVGYLFQD YALIPDRTVK  
FNL101QLAV EKHKWPEIPQ VLHAVGLESF EEKPVFELSG GEQQRATLAR  
VLLKNP151R IILADEPTGA LDLTNSELVI EALRALADKG ATVVVATHSP  
LFRESADT12 011KL

WARNING: possibly wrong combination

command: fasta

query: (Not FASTA Format)

database: nr-aa (Prot)

command	query	database
fasta	Prot	Prot
	Nucl	Nucl
tfasta	Prot	Nucl

FASTA searches a protein or DNA sequence data bank  
version 3.4t10 Dec 12, 2001

Please cite:

W. R. Pearson & D. J. Lipman PNAS (1988) 85:2444-2448

/bio/local/www/pub/tmp/fasta.68432364/fasta.tmp: 203 aa

>query

vs /bio/db/blast/db/nr-aa library

searching /bio/db/blast/db/nr-aa library

<	opt	E 0	
20	4820	0:==	
22	5	0:==	one = represents 2646 library sequences
24	25	1:*	
26	111	32:*	
28	804	349:*	
30	3193	2122:*	
32	9990	8204:***	
34	24842	22248:*****	
36	48798	45692:*****	
38	76872	75511:*****	
40	111112	105331:*****	
42	142638	128755:*****	
44	156662	142028:*****	
46	158731	144659:*****	
48	148286	138495:*****	
50	126410	126377:*****	
52	108134	111106:*****	
54	89128	94904:*****	
56	72856	79274:*****	
58	57683	65083:*****	
60	45073	52721:*****	
62	35897	42266:*****	
64	28047	33614:*****	
66	21218	26568:*****	
68	16380	20898:*****	
70	12373	16377:*****	
72	9488	12797:*****	

```

74 6958 9977:==*
76 5713 7766:==*
78 4244 6036:==*
80 3204 4687:==*
82 2443 3585:==*
84 1893 2840:==*
86 1362 2197:*
88 1129 1700:*      inset = represents 234 library sequences
90 742 1316:*
92 610 1018:*      :==*
94 474 788:*        :==*
96 359 609:*        :==*
98 274 472:*        :==*
100 269 365:*        :==*
102 175 282:*        :==*
104 122 218:*        :#
106 136 169:*        :#
108 109 131:*        :#
110 84 101:*         :#
112 59 78:*          :#
114 76 61:*          :#
116 64 47:*          :#
118 48 36:*          :#
>120 11700 28:===== :=====

```

500240239 residues in 1551842 sequences

statistics extrapolated from 60000 to 1540015 sequences

Expectation\_n flt: rho(ln(x))= 4.6919+/-0.000193; mu= 11.2022+/- 0.011

mean\_var=74.8953+/-16.395, 0's: 157 Z-trim: 407 B-trim: 2437 in 2/63

Lambda= 0.1482

Kolmogorov-Smirnov statistic: 0.0444 (N=29) at 50

FASTA (3.44 Dec 2001) function [optimized, /bio/db/fasta/matrix/aa/blosum50 matrix (15;-5)] ktup:

join: 36, opt: 24, open/ext: -10/-2, width: 16

Scan time: 267.910

The best scores are:

opt bits E(1551842)

Top 10  Select operation

<input checked="" type="checkbox"/>	pir:G95079 [G95079] ABC transporter, ATP-binding	( 213)	524	120	3.6e-26
<input checked="" type="checkbox"/>	prf:2504343R ORF - Streptococcus pneumoniae>tr:Q9	( 213)	521	120	5.6e-26
<input checked="" type="checkbox"/>	pir:B97947 [B97947] hypothetical protein ABC-NBD	( 213)	520	120	6.5e-26
<input checked="" type="checkbox"/>	prf:2805303ELE ABC transporter - Clostridium perf	( 211)	519	119	7.5e-26
<input checked="" type="checkbox"/>	tr:Q892J8 [Q892J8] Transporter.>gp:AE015948_111 [	( 212)	514	118	1.6e-25
<input checked="" type="checkbox"/>	pir:E95232 [E95232] ABC transporter, ATP-binding	( 213)	513	118	1.8e-25
<input checked="" type="checkbox"/>	pir:G95228 [G95228] ABC transporter, ATP-binding	( 210)	492	114	4.1e-24
<input checked="" type="checkbox"/>	prf:2713501E ATP-binding protein - Lactococcus la	( 207)	490	113	5.4e-24
<input checked="" type="checkbox"/>	pir:A95013 [A95013] hypothetical protein SP0111 [	( 213)	487	113	8.6e-24
<input checked="" type="checkbox"/>	pir:D97884 [D97884] hypothetical protein ABC-NBD	( 213)	486	112	1e-23
<input type="checkbox"/>	prf:2724351JF ABC transporter - Streptomyces aver	( 248)	480	111	2.7e-23
<input type="checkbox"/>	pir:B86626 [B86626] ABC transporter ATP-binding p	( 211)	474	110	5.9e-23
<input type="checkbox"/>	pir:D69433 [D69433] ABC transporter, ATP-binding	( 226)	466	108	2e-22
<input type="checkbox"/>	sp:Y065_MYCPN [P75612] Hypothetical ABC transport	( 465)	466	108	3.3e-22
<input type="checkbox"/>	tr:Q9LOJ9 [Q9LOJ9] Putative ABC-transporter ATP-b	( 246)	463	108	3.3e-22
<input type="checkbox"/>	pir:B69377 [B69377] ABC transporter, ATP-binding	( 228)	461	107	4.3e-22
<input type="checkbox"/>	prf:2719186AYG ABC transporter - Sulfolobus tokod	( 232)	460	107	5e-22
<input type="checkbox"/>	prf:2813338EKK MWP018 gene - Staphylococcus aureu	( 208)	459	107	5.4e-22
<input type="checkbox"/>	tr:Q8POU2 [Q8POU2] Putative ABC transporter (ATP-	( 233)	459	107	5.8e-22
<input type="checkbox"/>	pir:A84088 [A84088] ABC transporter (ATP-binding	( 228)	458	106	6.7e-22
<input type="checkbox"/>	pir:B97087 [B97087] ABC-type transport system AT	( 255)	457	106	8.3e-22

<input type="checkbox"/> tr:Q8RCC2 [Q8RCC2] ABC-type polar amino acid tran	( 240)	408	96 1.1e-18
<input type="checkbox"/> pir:AH1709 [AH1709] ABC transporter (ATP-binding	( 255)	408	96 1.2e-18
<input type="checkbox"/> pir:D97973 [D97973] hypothetical protein ABC-NBD	( 271)	408	96 1.2e-18
<input type="checkbox"/> tr:Q7P537 [Q7P537] ABC transporter ATP-binding pr	( 220)	407	95 1.2e-18
<input type="checkbox"/> tr:Q9CM47 [Q9CM47] Hypothetical protein PM0996.g	( 649)	412	97 1.3e-18
<input type="checkbox"/> tr:Q88F88 [Q88F88] ABC export system permease/AT	( 654)	412	97 1.3e-18
<input type="checkbox"/> tr:Q81K43 [Q81K43] ABC transporter, ATP-binding p	( 226)	407	96 1.3e-18
<input type="checkbox"/> trnew:AAR35564 [AAR35564] ABC transporter, ATP-bi	( 232)	407	96 1.3e-18
<input type="checkbox"/> tr:Q8PYB3 [Q8PYB3] ABC transporter, ATP-binding p	( 293)	408	96 1.3e-18
<input type="checkbox"/> sp:BCEA_BACSU [034697] Bacitracin export ATP-bind	( 253)	407	96 1.4e-18
<input type="checkbox"/> pir:AB1339 [AB1339] ABC transporter (ATP-binding	( 255)	407	96 1.4e-18
<input type="checkbox"/> prf:2805303ARP ABC transporter - Clostridium perf	( 255)	407	96 1.4e-18
<input type="checkbox"/> tr:Q92NU9 [Q92NU9] Probable transmembrane ATP-bin	( 647)	411	97 1.5e-18
<input type="checkbox"/> pir:D69858 [D69858] ABC transporter (ATP-binding	( 230)	406	95 1.5e-18
<input type="checkbox"/> pir:G96929 [G96929] ABC transporter ATP-binding p	( 238)	406	95 1.5e-18
<input type="checkbox"/> tr:Q8TQ83 [Q8TQ83] Lipoprotein releasing system	( 227)	405	95 1.7e-18
<input type="checkbox"/> pir:D69627 [D69627] cell-division ATP-binding pro	( 228)	405	95 1.7e-18
<input type="checkbox"/> pir:B86714 [B86714] hypothetical protein yhcA [im	( 664)	410	97 1.7e-18
<input type="checkbox"/> trnew:AAR34717 [AAR34717] ABC transporter, ATP-bi	( 234)	405	95 1.7e-18
<input type="checkbox"/> prf:2824301B bacitracin resistance-related protei	( 250)	405	95 1.8e-18
<input type="checkbox"/> trnew:AAT04916 [AAT04916] ABC transporter, ATP-bi	( 255)	405	95 1.9e-18
<input type="checkbox"/> tr:Q8G5S1 [Q8G5S1] Possible ATP binding protein o	( 263)	405	95 1.9e-18
<input type="checkbox"/> pir:T36431 [T36431] probable ABC-type transport s	( 264)	405	95 1.9e-18
<input type="checkbox"/> trnew:CAE50415 [CAE50415] Putative ABC transport	( 249)	404	95 2.1e-18
<input type="checkbox"/> tr:Q8DUD2 [Q8DUD2] Putative ABC transporter, ATP-	( 250)	404	95 2.1e-18
<input type="checkbox"/> tr:Q8PF15 [Q8PF15] ABC transporter ATP-binding pr	( 229)	403	95 2.3e-18
<input type="checkbox"/> sp:LOLD_EC0L6 [Q8FIM7] Lipoprotein releasing syst	( 233)	403	95 2.3e-18
<input type="checkbox"/> sp:LOLD_SH1EL [Q83RS0] Lipoprotein releasing syst	( 233)	403	95 2.3e-18
<input type="checkbox"/> trnew:CAF30191 [CAF30191] ABC transporter:ATPase	( 241)	403	95 2.4e-18
<input type="checkbox"/> pir:A83744 [A83744] ABC transporter (ATP-binding	( 260)	403	95 2.5e-18
<input type="checkbox"/> prf:2901400RM ABC transporter - Mycoplasma penetr	( 328)	404	95 2.6e-18
<input type="checkbox"/> pir:B97146 [B97146] ABC-type transport system, AT	( 224)	402	94 2.6e-18
<input type="checkbox"/> tr:Q884D4 [Q884D4] Macrolide ABC efflux protein.>	( 656)	407	96 2.7e-18
<input type="checkbox"/> tr:Q8R8L8 [Q8R8L8] Predicted ATPase involved in c	( 228)	402	94 2.7e-18
<input type="checkbox"/> prf:2824433GH ABC transporter - Oceanobacillus ih	( 228)	402	94 2.7e-18

>>pir:G95079 [G95079] ABC transporter, ATP-binding prote (213 aa)  
 initn: 438 initl: 284 opt: 524 Z-score: 613.5 bits: 120.5 E0: 3.6e-26  
 Smith-Waterman score: 524; 41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

10	20	30	40	50	60
query	MIEINDLKKSFGVRIWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPSSGQILV				
pir:G9	MIELKNISKKFGSRQLFSDMNLHFEGGKIYALIGTSGCGKTTLLNMIGRLEPYDKQIY				
10	20	30	40	50	60
70	80	90	100	110	
query	EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAV			EKKHWPEIPQ	
pir:G9	DGTSLKDI	KPSVFFRDYLGYLFDQFGLIESQTVKENLNLGLVGKKLKEKEKISLMKQ			
70	80	90	100	110	
120	130	140	150	160	170

ABC-Transporter





```

pir:E9 MIDIQGLEKKFNDRAIFSGNLKLEKGVYALIGKSGSGKTTLLNIGKLEKIDGGRVLY
      10      20      30      40      50      60
      70      80      90      100     110     120
query EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAVEKHKWPEIPQVLHAVG
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
pir:E9 QGKDLKTIPTRE--YFRDQMGYLFQNFGLLENQSIKENLDLGFVGQKISKVERLERQVG
      70      80      90      100     110
      130     140     150     160     170
query -LESFE-----EKPVFELSGGEQRTALARVLLKNPRIILADEPTGALDLTNSSELVIEA
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
pir:E9 ALEKVNGLYDLQEKIYTLSSGEAQRVALAKTILKNPPLILADEPTAALDPENSEEVMNL
      120     130     140     150     160     170
      180     190     200
query LRALADKGATVVVATHSPLFRESADTIIKL
      :::::  :::::  :::::  :::::  :::::  :::::
pir:E9 LVDLKDENRIIIATHNPLVMKADEIIDMRKLAHV
      180     190     200     210

```

>>pir:G95228 [G95228] ABC transporter, ATP-binding prote (210 aa)  
 initn: 500 initl: 270 opt: 492 Z-score: 576.6 bits: 113.6 E0: 4.1e-24  
 Smith-Waterman score: 492; 40.952% identity (43.216% ungapped) in 210 aa overlap (1-203:1-206)

```

      10      20      30      40      50      60
query MIEINDLKKSFQVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPSSGQILV
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
pir:G9 MIELKQVSKSFGERELFSNLSMTFEAGKVYALIGSSGSGKTTLMNMIGKLE-PYDGTIFY
      10      20      30      40      50
      70      80      90      100     110
query EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAVEKHKWPEIP-----Q
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
pir:G9 RGKDL--ANYKSSDFFRHELGYLFQNFGLIENQSIENLKLGLIGQKLSRSEQRLRQKQ
      60      70      80      90      100     110
      120     130     140     150     160     170
query VLHAVGLESFE-EKPVFELSGGEQRTALARVLLKNPRIILADEPTGALDLTNSSELVIEA
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
pir:G9 ALEQVGLVYLDLDRIFELSGGESQRVALAKIILKNPPFILADEPTASIDPATSQLIMEI
      120     130     140     150     160     170
      180     190     200
query LRALADKGATVVVATHSPLFRESADTIIKL
      :::::  :::::  :::::  :::::  :::::  :::::
pir:G9 LLSLRDDNRLIIATHNPAIWEMADEVFTMDHLK
      180     190     200     210

```

ABC  
transporter

>>prf:2713501E ATP-binding protein - Lactococcus lactis (207 aa)  
 initn: 422 initl: 236 opt: 490 Z-score: 574.3 bits: 113.2 E0: 5.4e-24  
 Smith-Waterman score: 490; 41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

```

      10      20      30      40      50      60
query MIEINDLKKSFQVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPSSGQILV
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
prf:27 MIELKNIEKSYDNHNIILHFNFYQKDNKSYALVGKSGSGKTTLLNIIIGRLPLDKGDIIL
      10      20      30      40      50      60
      70      80      90      100     110
query EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAV-----EKHKWPEI-PQ
      :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::  :::::
prf:27 DD-DNLK--TIPERRYFKOYLGYLFGNYGLIDNESIKDNLKLAFIGKKLKNQDQEIMSK
      70      80      90      100     110

```

ATP - ABC  
transporter

```

      120      130      140      150      160      170
query  VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSSELVIEA
      . . . . .
prf:27  ALSKVGLNENIDRKIFSLSGGEAQRVAIAKLIISKSPPIILADEPTGSLDRETGKEVMDI
      120      130      140      150      160      170

```

```

      180      190      200
query  LRALADKGATVVVATHSPLFRESADTI|KL
      . . . . .
prf:27  LLSLVKENTTVIIATHDSHVYNRVDSI|NL
      180      190      200

```

>>pir:A95013 [A95013] hypothetical protein SP0111 [Impor (213 aa)  
initn: 263 initl: 263 opt: 487 Z-score: 570.7 bits: 112.6 E0: 8.6e-24  
Smith-Waterman score: 487; 40.191% identity (41.379% ungapped) in 209 aa overlap (1-203:1-209)

```

      10      20      30      40      50      60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPSSGQILV
      . . . . .
pir:A9  MIELKNITKTIGGKVIDNLRLIDQGDVAIVGKSGSGKSTLLNLLGLIDGDYSGRYEI
      10      20      30      40      50      60

```

```

      70      80      90      100      110
query  EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAVEKHKWP-----IPQ
      . . . . .
pir:A9  FGQTNLAVNSAKSQTIIREHISYLFQNFALIDDETVEYNLMLALKYVKLPKKDKLKKVEE
      70      80      90      100      110      120

```

```

      120      130      140      150      160      170
query  VLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSSELVIEAL
      . . . . .
pir:A9  ILERVGLSATLHQRVSELSGGEQQRIVARAILKPSQLILADEPTGSLDPENRDLVLKFL
      130      140      150      160      170      180

```

```

      180      190      200
query  RALADKGATVVVATHSPLFRESADTI|KL
      . . . . .
pir:A9  LEMNREGKTVIIVTHDAYVAQQCHR|IELGEGK
      190      200      210

```

>>pir:D97884 [D97884] hypothetical protein ABC-NBD [impo (213 aa)  
initn: 263 initl: 263 opt: 486 Z-score: 569.6 bits: 112.4 E0: 1e-23  
Smith-Waterman score: 486; 39.713% identity (40.887% ungapped) in 209 aa overlap (1-203:1-209)

```

      10      20      30      40      50      60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPSSGQILV
      . . . . .
pir:D9  MIELKNITKTIGGKVIDNLRLIDQGDVAIVGKSGSGKSTLLNLLGLIDGDYSGRYEI
      10      20      30      40      50      60

```

```

      70      80      90      100      110
query  EDVDLLKLSTRKQRLYRKNTVGYLFDQYALIPDRTVKFNLQLAVEKHKWP-----IPQ
      . . . . .
pir:D9  FGQTNLAVNSAKSQTIIREHISYLFQNFALIDDETVEYNLMLALKYVKLPKKDKLKKVEE
      70      80      90      100      110      120

```

```

      120      130      140      150      160      170
query  VLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSSELVIEAL
      . . . . .
pir:D9  ILERVGLSATLHQRVSELSGGEQQRIVARAILKPSQLILADEPTGSLDPENRDLVLKFL
      130      140      150      160      170      180

```

```

      180      190      200
query  RALADKGATVVVATHSPLFRESADTI|KL
      . . . . .

```

putative  
ABC transporter



pir:D9 LEMNREGKTVIIVTHDAYVAQQCHRVIELGEGK  
190 200 210

>>prf:2724351JF ABC transporter - Streptomyces avermitil (248 aa)  
initn: 308 initl: 255 opt: 480 Z-score: 561.8 bits: 111.2 E0: 2.7e-23  
Smith-Waterman score: 480; 40.845% identity (43.500% ungapped) in 213 aa overlap (2-203:9-219)

query MIEINDLKKSFG---VRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLG  
prf:27 MGQMSNDALQLRSVSRRYGAGGGAVTALDQ-VSLAFPRGTFTAVMGPSGSGKSTLLQCAA  
10 20 30 40 50

query 50 60 70 80 90 100  
TLDKPSSGQILVEDVLLKSTRKQRLYRKNTVGylFQDYALIP---DRTVKFNQLAV  
prf:27 GLDRPTSGSVTVGDTLTKLSETKLTLLRRDRIGFVFOAFNLLPSLTAEQNVALPLRLAG  
60 70 80 90 100 110

query 110 120 130 140 150 160  
EKHKWPEIPQVLHAYGL-ESFEEKPVFELSGGEQRTALARVLLKNPRIILADEPTGALD  
prf:27 RRPRTKEVREVLAVGLGDRAGHRPT-EMSGGQQQRVALARALITRPDVLFGDEPTGALD  
120 130 140 150 160 170

query 170 180 190 200  
LTNSELVIEALRALAD-KGATVVVATHSPLFRESADTI IKL  
prf:27 SQTsREVLTLRGMVDSEGQTVIMVTHDPVAASYADRVVFLVDGRVNGELIGASAEDIAA  
180 190 200 210 220 230

prf:27 RMTKLEAPC  
240

>>pir:B86626 [B86626] ABC transporter ATP-binding protei (211 aa)  
initn: 283 initl: 283 opt: 474 Z-score: 555.8 bits: 109.8 E0: 5.9e-23  
Smith-Waterman score: 474; 39.234% identity (40.394% ungapped) in 209 aa overlap (1-203:1-209)

query MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPKSSGQILV  
pir:B8 MIEIEELTKSYKGHIIFDKLNLRIPEGKMTAITYTSGAGKSTLLNIGLIEDYDDGKYFF  
10 20 30 40 50 60

query 70 80 90 100 110  
EDVLLKSTRKQRLYRKNTVGylFQDYALIPDRTVKFNQLAV---EKHKWPEIPQ  
pir:B8 NGQFAPPFNSSLALKMRRNKISYLFQNFALLEDETEKNLEIALIYSRISKKEKRKKMKK  
70 80 90 100 110 120

query 120 130 140 150 160 170  
VLHAYGLESFEEKPVFELSGGEQRTALARVLLKNPRIILADEPTGALDLTNSELVIEAL  
pir:B8 LLLQVGINHLNLTQVYSLSGGEKQRVAIARALLKESQLILADEPTGSLDTENRNEVIALL  
130 140 150 160 170 180

query 180 190 200  
RALADKGATVVVATHSPLFRESADTI IKL  
pir:B8 RQEVDKGKAVVIVTHDSYLKEVSDLVIEIGE  
190 200 210

>>pir:D69433 [D69433] ABC transporter, ATP-binding prote (226 aa)  
initn: 436 initl: 242 opt: 466 Z-score: 546.1 bits: 108.1 E0: 2e-22  
Smith-Waterman score: 466; 39.583% identity (41.081% ungapped) in 192 aa overlap (19-203:25-216)

```

      10      20      30      40      50
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPKS
pir:D6 MKVIELVDVYKIYRTAYYEVHALDGVSMEVEAGEFVAIMGPSGSGKSTLLNMIGCLDKPT
      10      20      30      40      50      60

      60      70      80      90      100
query  SGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQL——AVEKHK
pir:D6 KGEVIINGVKTSGLSDRELTKLRDSIGFIFQQYNLIPTLTALENVELPMIFRGVARAER
      70      80      90      100      110      120

      110      120      130      140      150      160
query  WPEIPQVLHAVGLESFEEKPVFELSGGEQRTALARVLLKNPRIILADEPTGALDLTNSE
pir:D6 ERRAKELLKVVGIEELADRRPREMSGGQQQRAIARALANNPKILLCDEPTGNLDTKSGR
      130      140      150      160      170      180

      170      180      190      200
query  LVIEALRALADK-GATVVVATHSPLFRESADTIILK
pir:D6 QVMGILKNLNEENGVTVVLVTHDPSLSEYADRVIRIRDGKVVEDVY
      190      200      210      220

```

>>sp:Y065\_MYCPN [P75612] Hypothetical ABC transporter AT (465 aa)  
 initn: 286 init1: 243 opt: 466 Z-score: 542.2 bits: 108.4 E0: 3.3e-22  
 Smith-Waterman score: 466; 36.667% identity (38.308% ungapped) in 210 aa overlap (1-201:231-440)

```

      10      20
query  MIEINDLKKSFGVRILWQGLSHKFLP
sp:Y06 HFLKNEVKKVTWLNPRAKKESVTPDEEHI IELKNVYKYITNGVTTNAVVKGIDLKKA
      210      220      230      240      250      260

      30      40      50      60      70      80
query  GTMTALTGASGSGKSTLLNCLGTLDPKSSGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQ
sp:Y06 HDFIVILGPSGSGKTLLNIIISGMDRPSGSGSVVNGQEMICMNDRQLTNFRNRYVGYIFQ
      270      280      290      300      310      320

      90      100      110      120      130      140
query  DYALIPDRTVKFNLQAVEKHKWPE——IPQVLHAVGLESFEEKPVFELSGGEQRTAL
sp:Y06 QYGLLPNLTVRENVEVGANLQRNPKRINIDELLEAVGMKHLQKKLPNELSGGQQQVSI
      330      340      350      360      370      380

      150      160      170      180      190      200
query  ARVLLKNPRIILADEPTGALDLTNSELVIEALRALADK-GATVVVATHSPLFRESADTIIL
sp:Y06 ARAFAKNPLLIFGDEPTGALDLEMTQIVLKQFLAKQRYKTTMVI VTHNNLI AQLADLVI
      390      400      410      420      430      440

```

query KL

sp:Y06 YVADGKIQALQANPNPKQVEDINWI  
 450 460

>>tr:Q9L0J9 [Q9L0J9] Putative ABC-transporter ATP-bindin (246 aa)  
 initn: 311 init1: 233 opt: 463 Z-score: 542.2 bits: 107.5 E0: 3.3e-22  
 Smith-Waterman score: 463; 44.262% identity (46.023% ungapped) in 183 aa overlap (27-203:36-217)

```

      10      20      30      40      50
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDPKSSG

```

ABC  
transporter

```

      10      20      30      40      50      60
tr:Q9L A I R L S S V S R R Y G A G E G T V T A L D D V S L A L R R G S F T A V M G P S G S G K S T L L Q C A A G L D R P T S G
      60      70      80      90      100     110
query  Q I L V E D V D L L K L S T R K Q R L Y R K N T V G Y L F Q D Y A L I P D R T — V K F N L Q L A V E K H K W P E I
      10      20      30      40      50      60
tr:Q9L S V V V G G T E L T G L S Q R R L T L L R R E R V G F V F Q A F N L L P S L T A A Q N V A L P L R L A G R R P P R G R V
      70      80      90      100     110     120
      120     130     140     150     160     170
query  P Q V L H A V G L — E S F E E K P V F E L S G G E Q Q R T A L A R V L L K N P R I I L A D E P T G A L D L T N S E L V I
      10      20      30      40      50      60
tr:Q9L R E A L R Q V G L A D R A R H R P A — E L S G G Q Q R V A L A R A L I T R P Q V L F A D E P T G A L D S R T G R E V L
      130     140     150     160     170     180
      180     190     200
query  E A L R A L A D — K G A T V V V A T H S P L F R E S A D T I I K L
      10      20      30      40      50
tr:Q9L T L L R A M A D G E G R T V V M V T H D P V A A S Y A D R V L F L V D G R V H D E L T G S G P D G I A T R M T R L E A A
      190     200     210     220     230     240
tr:Q9L P C

```

>>pir:B69377 [B69377] ABC transporter, ATP-binding prote (228 aa)  
 initn: 483 initl: 234 opt: 461 Z-score: 540.3 bits: 107.1 E0: 4.3e-22  
 Smith-Waterman score: 461; 39.000% identity (40.625% ungapped) in 200 aa overlap (11-203:18-216)

```

      10      20      30      40      50
query  M I E I N D L K K S F G V R I L W Q G L S H K F L P G T M T A L T G A S G S G K S T L L N C L G T L D K P
      10      20      30      40      50
pir:B6 M K V V E L R N V Y K I Y R T E Y Y E V R A L — D G V S M D V E E G E F V V I M G P S G S G K S T L L N I G C L D K P
      60      70      80      90      100
query  S S G Q I L V E D V D L L K L S T R K Q R L Y R K N T V G Y L F Q D Y A L I P D R T V K F N L Q L A V — E K H
      60      70      80      90      100     110
pir:B6 T E G E V L I N G V E T S S L N D N R L T E L R R D T I G F I F Q T Y N L I P T L T A L E N V E L P M I F K G V G R R E
      110     120     130     140     150     160
query  K W P E I P Q V L H A V G L E S F E E K P V F E L S G G E Q Q R T A L A R V L L K N P R I I L A D E P T G A L D L T N S
      120     130     140     150     160     170
pir:B6 R E E R A K E L L K N V G L E K E M N R K P N E M S G G Q Q R V A I A R A L A N N P K I L L C D E P T G N L D T K S G
      170     180     190     200
query  E L V I E A L R A L A D K — G A T V V V A T H S P L F R E S A D T I I K L
      180     190     200     210     220
pir:B6 E Q V M E I I R H Q N E V L G V T V I L V T H D P S L A K Y G D R V I R L R D G K I E S V E N V S

```

>>prf:2719186AYG ABC transporter - Sulfolobus tokodali>t (232 aa)  
 initn: 415 initl: 240 opt: 460 Z-score: 539.1 bits: 106.8 E0: 5e-22  
 Smith-Waterman score: 460; 35.681% identity (37.811% ungapped) in 213 aa overlap (1-201:6-218)

```

      10      20      30      40      50
query  M I E I N D L K K S F G V R I L — W Q G L S H K F L P G T M T A L T G A S G S G K S T L L N C L G T L D
      10      20      30      40      50     60
prf:27 M S E D E L I I I E N L K K I Y K I N V E F P A L R G I N L K I Y K G E F L G I A G P S G S G K T T L L D M I G L L D
      60      70      80      90      100     110
query  K P S S G Q I L V E D V D L L K L S T R K Q R L Y R K N T V G Y L F Q D Y A L I P D R T V K F N L Q L A V E K H K W P —

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## The Reference 2

### ORIGINAL PAPER

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## A transporter of *Escherichia coli* specific for L- and D-methionine is the prototype for a new family within the ABC superfamily

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**Abstract** An ABC-type transporter in *Escherichia coli* that transports both L- and D-methionine, but not other natural amino acids, was identified. This system is the first functionally characterized member of a novel family of bacterial permeases within the ABC superfamily. This family was designated the methionine uptake transporter (MUT) family (TC #3.A.1.23). The proteins that comprise the transporters of this family were analyzed phylogenetically, revealing the probable existence of several sequence-divergent primordial paralogues, no more than two of which have been transmitted to any currently sequenced organism. In addition, MetJ, the pleiotropic methionine repressor protein, was shown to negatively control expression of the operon encoding the ABC-type methionine uptake system. The identification of MetJ binding sites (in gram-negative bacteria) or S-boxes (in gram-positive bacteria) in the promoter regions of several MUT transporter-encoding operons suggests that many MUT family members transport organic sulfur compounds.

**Electronic Supplementary Material** Supplementary material is available for this article if you access the article at <http://dx.doi.org/10.1007/s00203-003-0561-4>. A link in the

frame on the left on that page takes you directly to the supplementary material.

**Keywords** Transport · Methionine · MetD · ATP-binding cassette · *E. coli*

### Introduction

Methionine transport and its regulation have been extensively studied in both *Escherichia coli* and the phylogenetically related bacterium *Salmonella typhimurium*. *E. coli* has been shown to have two transport systems for L-methionine (Kadner 1974) but only one system for D-methionine (Kadner 1977). Spontaneous mutants selected for their capacity to grow on toxic methionine analogues were generated. A *metD* mutant lacks both high-affinity uptake activity for L-methionine (Kadner 1974) and lower affinity uptake activity for D-methionine (Kadner 1977; Kadner and Watson 1974). Specificity of the transport system for L- and D-methionine and related compounds has been examined (Kadner 1974, 1977), but inhibitory studies with other amino acids have not been reported. A *metD* mutant has also been isolated in *S. typhimurium* and exhibits characteristics similar to those of the corresponding mutant in *E. coli* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Studies of energy coupling for methionine uptake in *E. coli* have suggested that transport is driven by phosphate bond energy, presumably ATP (Kadner and Winkler 1975). Moreover, the MetD transport system has been shown to be sensitive to osmotic shock and to inhibition by arsenate in both *E. coli* and *S. typhimurium* (Cottam and Ayling 1989; Kadner and Winkler 1975). It was therefore suggested that the major uptake system is an ATP-binding cassette (ABC) transporter. ABC transporters usually consist of a transmembrane protein, a cytoplasmic ATP-hydrolyzing (ABC) protein, and at least one substrate-binding receptor.

The *metD* mutation was mapped to 4.8 minutes on the *E. coli* chromosome (Berlyn 1998). Mapping of the *metD*

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locus in *S. typhimurium* has revealed that several genes, when mutated, give rise to the *metD* phenotype (Grundy and Ayling 1992). In the work reported here, we identify an ABC transporter mapping very near 4.8 minutes, which proves to be the *MetD* transporter.

Methionine biosynthetic genes in *E. coli* are regulated by the *MetJ* repressor (Greene 1996; Sekowsha et al. 2000; Weissbach and Brot 1991), and a *MetJ* DNA binding site consensus sequence has been derived (Saint-Girons et al. 1984). Evidence is available suggesting that methionine transport is also regulated by *MetJ* since cells grown in medium containing methionine have lower levels of methionine transport (Kadner 1975), and methionine auxotrophs with a *metJ* mutation have higher transport activity (Kadner 1975, 1977). The recent identification of a *MetJ* binding site in the promoter region of the ABC transporter mentioned above (Liu et al. 2001) corroborated this hypothesis.

Recently, Gal et al. (2002) and Merlin et al. (2002) reported growth studies that led to the tentative molecular identification of this transporter (*abc-yaeE-yaeC*) which they renamed *metNIQ*. The *MetJ* protein, in the presence of methionine, but not in its absence, was also shown to repress expression of the operon. Although no transport studies have been reported, it has been suggested that the three-gene cluster encodes the methionine transporter characterized physiologically by Kadner and his collaborators.

In this study, we confirm and substantially extend the previously reported work. We (1) report the first transport studies with this system, revealing that *MetD* transports both L- and D-methionine and probably formyl methionine; (2) resolve the question as to whether both L- and D-methionine are recognized by the same receptor; (3) provide evidence that *NlpA* (Yamaguchi and Inouye 1988; Yu et al. 1986) may, under certain conditions, serve as a poor L- and D-methionine receptor, feeding inefficiently into *MetD*; (4) show that *MetJ* represses expression of the

*metD* operon; and (5) provide detailed phylogenetic data that define a novel family within the ABC superfamily, which we have called the methionine uptake transporter (MUT) family (TC #3.A.1.23).

## Materials and methods

### Bacterial strains and media

*E. coli* strains and plasmids used in this study are listed in Table 1. All studies were conducted in the genetic background of strain BW25113. Bacteria were cultured in Luria-Bertani (LB) broth or M9 minimal medium at 37°C (Sambrook et al. 1989). When appropriate, ampicillin (Ap) and/or kanamycin (Km) was/were added to the medium at 100 and/or 25 µg/ml, respectively. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich.

### Generation of deletion mutants

Deletion mutants were generated using the methods described by Datsenko and Wanner (2000). To prepare competent cells for transformation, BW25113 containing pKD46 was cultured at 30°C in SOB broth (Sambrook et al. 1989) containing ampicillin and 2 mM L-arabinose. When the OD<sub>600</sub> reached 0.5, the culture was centrifuged at 2,000×g for 5 min, and the cells were washed three times with cold 10% glycerol before being resuspended in a minimal volume of 10% glycerol (1% of the original culture). The competent cells were stored at -80°C prior to use. PCR methods were used to clone the kanamycin gene from pKD4 using the primers described in Table 2. The PCR products were purified using a QIAGEN kit, treated with *DpnI*, and repurified by electrophoresis. BW25113 competent cells were transformed with the kanamycin gene by electroporation (Gene Pulser, pulse controller at 200 Ω, capacitance at 250 µF, and voltage at 2.5 kV). After electroporation, the cells were grown with shaking in 1 ml of SOC (Sambrook et al. 1989) at 37°C for 1 h, and the cultures were plated onto LB agar medium containing kanamycin. The kanamycin-resistant transformants were purified on new kanamycin-LB plates. The mutants in which the target genes were replaced by the kanamycin gene were verified by three PCRs using bacterial DNA as the template. The first PCR used forward primer k<sub>2</sub> (5'-CGGTGCCCTGAATGAACCTGC-3') and reverse primer k<sub>1</sub> (5'-CGGCCACAGTCGATGAATCC-3') (Datsenko and Wanner 2000), both of

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype	Reference
<b>Strain</b>		
LJ 3001 (BW25113)	<i>lacMrrnB<sub>714</sub> ΔlacZ<sub>W110</sub> hsdR514 ΔaraBAD<sub>AN35</sub> ΔrhaBAD<sub>LD74</sub></i>	Datsenko and Wanner 2000
LJ 3015	BW25113 <i>Δabc-yaeE (ΔmetNI)</i>	This study
LJ 3016	BW25113 <i>ΔyaeC (ΔmetQ)</i>	This study
LJ 3017	BW25113 <i>ΔnlpA</i>	This study
LJ 3018	BW25113 <i>ΔyaeC ΔnlpA</i>	This study
LJ 3019	BW25113 <i>ΔyaeE-abc dykJD (ΔmetNI ΔmnuP)</i>	This study
LJ 3020	BW25113 <i>ΔykdD</i>	This study
LJ 3021	BW25113 <i>ΔmetJ</i>	This study
<b>Plasmid</b>		
pKD46	<i>oriR101 repA101(ts) araBp-gam-bet-exo Ap<sup>r</sup></i>	Datsenko and Wanner 2000
pKD4	<i>oriR<sub>1</sub> Ap<sup>r</sup> Km<sup>r</sup></i>	Datsenko and Wanner 2000
pCP20	<i>Δc1857(ts) ts-rep</i>	Datsenko and Wanner 2000
pBAD24	Expression vector, Ap <sup>r</sup>	Guzman et al. 1995
pBAD24- <i>metD</i>	pBAD24 carrying <i>metD</i> operon	This study
pBAD24- <i>metJ</i>	pBAD24 carrying <i>metJ</i>	This study

Table 2 Primers used for generation and verification of BW25113 mutants. For all gene mutations, pKD4 was used as the template for cloning the kanamycin gene

Gene	Primers (5'→3')
<i>abc-yaeE (metN)</i>	Generation GATGCGGTCGCCTGCGAACTGAATTAATAAAACCAGAAATGACCAGGTGTAGGCTGGAGCTGCTTC (forward) CTTAATGACGATATAAAATAATCAATGATAAACTTTGCAATATCCATATGAATATCCTCCTTAG (reverse) Verification <i>abc-yaeE</i> 3: CGTTACTTGCAGGTGACAGC <i>abc-yaeE</i> 4: GCATGTGACGCTAGTATCGC
<i>yaeC (metQ)</i>	Generation TTACAAATTGTGGAAACAGCCTAAAAATTACCAGCCTTTAACAGCGTGTAGGCTGGAGCTGCTTC (forward) AAGGAATAAGGTATGGCGTTCAAATTCAAAACCTTTGCGGCAGTG CATATGAATATCCTCCTTAG (reverse) Verification <i>yaeC</i> 3: ACAGCCGCTTAGCATGAGTG <i>yaeC</i> 4: AATTCAGTTCGACGGCGACC
<i>nlpA</i>	Generation ACCGCAGCGACCTTACCGCTATAGTCAGGTAATCATTAAATAAAAGGTGTAGGCTGGAGCTGCTTC (forward) TGAGAATTACCAGCCAGGCACCGCGCCACCGTTAAAAATGGTTTCCATATGAATATCCTCCTTAG (reverse) Verification <i>nlpA</i> 3: CGTGGTCAGTAAGAAGTGCC <i>nlpA</i> 4: GCTGCTGATTCTGTCATCGG
<i>ykdD (metU)</i>	Generation GOTTGACTTTGCATTCTGTTAACAAACGCGGTATAACAAACCGTGTAGGCTGGAGCTGCTTC (forward) GOTTGAGTAAGGAAATAAGCACCATAGCACAAACGCAACAAACCATATGAATATCCTCCTTAG (reverse) Verification <i>ykdD</i> 3: GACTTGTTCGCACCTTCC <i>ykdD</i> 4: GGCTGTGCGCTAAGTTAC
<i>metJ</i>	Generation TGGTCTGGTCTCAATTTATTGACGAAGAGGATTAAGTATCTCATGGTGTAGGCTGGAGCTGCTTC (forward) TAGCGCATCAGCGGATTCCACTCGCGCGCTCTTTTGTCTTACATATGAATATCCTCC AATG (reverse) Verification <i>metJ</i> 3: CAACTGTGTGGTCTGGTCTC <i>metJ</i> 4: TGCGATGACGAGAGATCTG

which are designed from the internal kanamycin gene. The second PCR used primer 2 and primer 4 (reverse, Table 2). The third PCR used primers 3 and 4 (Table 2). To delete the kanamycin gene from the chromosome, pKD46 was removed from the cells by growing the bacteria at 37 °C, and then pCP20 was introduced by transformation. The transformants containing pCP20 were grown overnight with shaking at 42 °C, and the cultures were plated on LB agar without antibiotics. Colonies were tested for sensitivity to kanamycin and ampicillin. To verify the loss of the kanamycin gene, the last PCR using primers 3 and 4 (see above, Table 2) was repeated. Growth experiments were carried out in M9 minimal medium, with MgCl<sub>2</sub> replacing MgSO<sub>4</sub> and with 20 μM L-methionine or 100 μM D-methionine serving as the sole sulfur source.

#### DNA manipulations and gene cloning

Standard methods were used for chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Sambrook et al. 1989). Plasmids were isolated using spin miniprep kits (Qiagen, Chatsworth, Calif., USA), and PCR products were purified using Qiaquick purification kits (Qiagen). For gene cloning, the *metD* operon (*abc-yaeE-yaeC*) and *metJ* gene were amplified from wild-type *E. coli* BW25113 chromosomal DNA by PCR. The following primers were used for gene amplification (restriction sites *KpnI*, *XbaI* and *HindIII* are underlined) – for *metD*, 5'-C G C G G T A C C G A T A A A C T T T C G

A A T A T C A C C -3' and 5'-C G C C T C T A G A T T A C A A A T T G T G G A A A C A G C C -3', and for *metJ*, 5'-G A A T C T A G A C A T G G C T G A A T G G A G C G G C G -3' and 5'-C C C C A A G C T T A G T A T T C C C A C G T C T C C G -3'. The PCR products were purified, treated with *KpnI* and *XbaI* (for *metD*) or with *XbaI* and *HindIII* (for *metJ*), and then cloned into pBAD24 (Guzman et al. 1995).

#### Transport assays

Cells grown in M9 minimal medium were harvested in the exponential growth phase, washed once in Tris-maleate (TM) buffer, pH 7.0, and resuspended in the same buffer containing 0.5% D,L-lactate plus 100 μg chloramphenicol/ml. Uptake studies were conducted at 37 °C over a 10-min time interval with the cell density at an OD<sub>600</sub> of 0.10 and 0.40 and the methionine concentration at 0.5 μM (50,000 cpm/ml; 55 μCi/μmol) and 5.0 μM (500,000 cpm/ml; 5.5 μCi/μmol), respectively, for the L- and D-isomers. 1-[<sup>14</sup>C]L- and D-methionine were purchased from American Radiolabeled Chemicals. Aliquots (100 μl) were periodically removed from the 1-ml cell suspensions. Cells were transferred to 10 ml of ice-cold TM buffer, filtered (0.45-μm Millipore filters) and washed twice with the same buffer. After drying the filters, radioactivity was measured by scintillation counting using 10 ml of Biosafe NA scintillation fluid (Research Products International, Mt. Prospect, Ill., USA). For comparison of the uptake of L-methionine between

the *abc-yacE* and the *abc-yacE mmuP* mutants, L-methionine concentrations of 13 and 103  $\mu\text{M}$  (12  $\mu\text{Ci}/\mu\text{mol}$ ) were used since the low-affinity L-methionine transporter was analyzed. Initial uptake rates were inhibited over a 5-min time interval for both L- and D-methionine. Unless otherwise stated, the concentration of the non-radioactive inhibitory amino acid was ten times the methionine concentration. The uptake activity attributed to MetD alone was obtained by subtracting the activity remaining in the absence of MetD function from the wild-type activity (e.g., see Table 3), assuming that loss of MetD does not activate some other transporter.

#### Computer methods

Sequences of the proteins that comprise the three constituents of MUT family permeases were obtained by initial BLAST searches (Altschul et al. 1997) using the sequences of the three *E. coli* MetD permease constituents as query. The resulting hits were filtered through a program manipulating the BLAST program to eliminate the sequences more related to other families in the ABC superfamily (C. Tran and M.H. Saier, Jr., unpublished program).

Multiple sequence alignments were constructed using the Clustal X program (Thompson et al. 1997). The gap penalty and gap extension values used with the Clustal X program were 10 and 0.1, respectively, although other combinations were tried. The HMMTOP (Tusnady and Simon 1998, 2001) and TMHMM (Krogh et al. 2001; Sonnhammer et al. 1998) programs were used to determine the predicted numbers of transmembrane segments. Phylogenetic trees were derived from alignments generated with the Clustal X program using the BLOSUM 62 scoring matrix. The trees were drawn using the TreeView program (Page 1996). Complementary trees were constructed using the Phylo\_win program (Galtier et al. 1996) with the neighbor-joining method and FAM distances as the model of evolution. This study was conducted independently for the three protein constituents of the ABC transporters that comprise the MUT family, and the sequences obtained were checked manually to see whether all three ABC elements had been identified for each transporter.

G+C content was analyzed with the GeoCec program (Rice et al. 2000), and codon usage was analyzed with the Countcodon program from the Codon Usage Database website (<http://www.kazusa.or.jp/codon/countcodon.html>). The lipoprotein structure of the receptors was predicted with the Lipop section of the PSORT program (<http://psort.nibb.ac.jp>). S-boxes were predicted with the RNAPattern program (Vitreschak et al., unpublished program).

## Results

### Growth studies

Figure 1 shows the growth of the isogenic strains described above with 100  $\mu\text{M}$  D-methionine as the sole source of sulfur. The wild-type strain, the *nlpA* mutant, and the *ykfD* (Thanbichler et al. 1999) mutant grew equally well, but the *abc-yacE* mutant and the *yacC nlpA* double mutant as well as the *abc-yacE-ykfD* triple mutant grew very poorly. Both the growth rates and growth yields were substantially depressed. These effects were completely reversed by inclusion of a plasmid (pBAD24) bearing the *metD* (*abc-yacE-yacC*) operon (data not shown, see supplementary Fig. S1 in the electronic supplementary material). The *yacC* single mutant grew substantially better than the two double mutants but much less well than the wild-type strain. When 20  $\mu\text{M}$  L-methionine served as the sole source of sulfur, the difference between the wild-type and the double mutants was less pronounced than when D-methionine

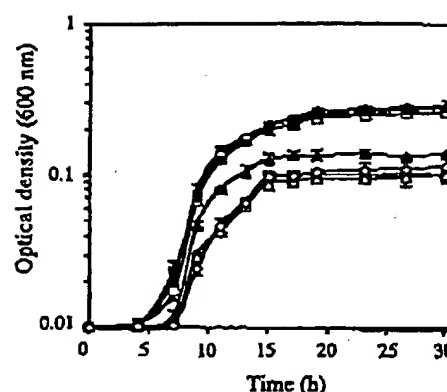


Fig. 1 Growth of *Escherichia coli* as a function of time in M9 minimal medium in which  $\text{MgSO}_4$  was replaced by 1 mM  $\text{MgCl}_2$  and D-methionine was added at a concentration of 100  $\mu\text{M}$  as the sole sulfur source present. The growth experiments were conducted three times, and the results were averaged. The following strains were examined: wild-type (BW25113) ( $\blacklozenge$ ),  $\Delta abc-yacE$  ( $\blacksquare$ ),  $\Delta yacC$  ( $\blacktriangle$ ),  $\Delta nlpA$  ( $\bullet$ ),  $\Delta yacC-\Delta nlpA$  ( $\diamond$ ),  $\Delta ykdD$  ( $\square$ ) and  $\Delta abc-yacE \Delta ykdD$  ( $\Delta$ ). Error bars indicate standard deviations.

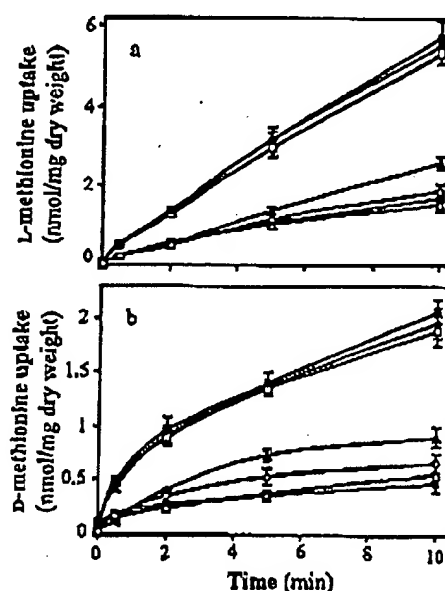


Fig. 2 Uptake of 1-[ $^{14}\text{C}$ ]L-methionine (a) and 1-[ $^{14}\text{C}$ ]D-methionine (b) by wild-type (BW25113) ( $\blacklozenge$ ),  $\Delta abc-yacE$  ( $\blacksquare$ ),  $\Delta yacC$  ( $\blacktriangle$ ),  $\Delta nlpA$  ( $\bullet$ ),  $\Delta yacC-\Delta nlpA$  ( $\diamond$ ),  $\Delta ykdD$  ( $\square$ ) and  $\Delta abc-yacE \Delta ykdD$  ( $\Delta$ ) cells. Cells grown in M9 minimal medium were prepared as described in Materials and methods. Uptake assays were carried out at 37  $^{\circ}\text{C}$  over a 10-min time interval with the optical densities (600 nm) at 0.1 (a) or 0.4 (b) and the methionine concentrations at 0.5  $\mu\text{M}$  (55  $\mu\text{Ci}/\mu\text{mol}$ ) or 5  $\mu\text{M}$  (5.5  $\mu\text{Ci}/\mu\text{mol}$ ) (both at 50,000 cpm/ml), respectively, for the L- or D-isomers. Values are expressed in pmol L- or D-methionine retained per mg bacterial dry weight. The experiment was conducted three times, and the results reported represent an average of the values obtained. Error bars indicate standard deviations.

was used (Fig. 1; data not shown). However, depressed growth suggested that the transporter could accept both D- and L-methionine as substrates.

### Transport studies

Figure 2 shows the uptake of [ $^{14}$ C]L-methionine (Fig. 2a, 0.5  $\mu$ M) and [ $^{14}$ C]D-methionine (Fig. 2b, 5  $\mu$ M), respectively. Relative rates of uptake of the two substrates by the wild-type and mutant strains were essentially the same. Thus, the wild-type, and the mutant strains *ylgD* and *nlpA* took up the amino acids at nearly the same rate; the two double mutants (*abc-yaeE* and *yaeC nlpA*) took up both substrates poorly, and the *yaeC* single mutant took up both substrates poorly but slightly better than the double mutants. These depressed uptake rates were largely reversed by inclusion of the plasmid-encoded *metD* operon in these strains (data not shown; see Fig. S2 in the electronic supplementary material). It was therefore concluded that: (1) both L- and D-methionine are substrates of the *Abc-YaeE-YaeC* transporter, and (2) the binding receptor *YaeC*, and possibly *NlpA*, can activate both D- and L-methionine uptake. If *NlpA* acts as a receptor for the

*Abc-YaeE* system, it is a much less effective receptor than *YaeC*. For both isomers, residual uptake was observed in the *abc-yaeE* mutant, suggesting that a second transporter exists both for L- and for D-methionine. We tested the transport of L- and D-methionine at concentrations of 10 and 100  $\mu$ M in the *mmuP abc-yaeE* triple mutant, but no significant difference was observed relative to the *abc-yaeE* mutant (data not shown). This result is consistent with the indistinguishable growth rates between the *abc-yaeE-yaeC* and the *abc-yaeE-yaeC mmuP* mutants observed by Gal et al. (2002) in a methionine auxotrophic strain grown in minimal medium supplemented with L-methionine. It can therefore be assumed that the S-methylmethionine permease *MmuP* is not responsible for the residual transport of L- or D-methionine observed in the *abc-yaeE* mutant.

### Inhibition studies

Table 3 summarizes the inhibitory effects of several non-radioactive amino acids present at ten-fold the concentration of the radioactive amino acid on uptake of both L- and D-methionine. Uptake of L-methionine by the wild-type bacteria was strongly inhibited by L-methionine and weakly

**Table 3** Inhibition of L-methionine (top) and D-methionine (bottom) uptake by the L- and D-isomers of several amino acids. Assays were done as described in Materials and methods with the non-radioactive inhibitory amino acids at 10x the concentrations of the radioactive substrate. Rate of uptake is expressed in pmol/(minxmg dry weight). Wild type-*Abc-yaeE* is the uptake activity attributed to *MetD* alone

Inhibitor	Wild-type		<i>Abc-yaeE</i>		Wild-type- <i>Abc-yaeE</i>	
	Rate	%	Rate	%	Rate	%
—	688.8 $\pm$ 88	100	230.4 $\pm$ 16	100	458.5 $\pm$ 72	100
L-Methionine	47.5 $\pm$ 5	7	41.8 $\pm$ 8	18	5.7 $\pm$ 3	1
D-Methionine	635.3 $\pm$ 45	92	298.6 $\pm$ 16	129	336.8 $\pm$ 30	73
N-Formyl-L-methionine	268.3 $\pm$ 18	39	258.9 $\pm$ 13	112	9.3 $\pm$ 5	2
L-Alanine	627.4 $\pm$ 56	91	176.9 $\pm$ 20	77	451.1 $\pm$ 35	98
D-Alanine	649.5 $\pm$ 40	94	281.6 $\pm$ 22	122	367.9 $\pm$ 18	80
L-Leucine	581.8 $\pm$ 40	84	179.7 $\pm$ 16	78	402.1 $\pm$ 24	88
D-Leucine	677.5 $\pm$ 56	98	245.6 $\pm$ 36	107	431.9 $\pm$ 20	94
L-Valine	528.1 $\pm$ 50	77	159.1 $\pm$ 18	69	369.0 $\pm$ 31	80
L-Serine	833.2 $\pm$ 105	121	271.7 $\pm$ 17	118	561.5 $\pm$ 88	122
D-Serine	590.3 $\pm$ 57	86	304.8 $\pm$ 25	132	285.5 $\pm$ 34	62
L-Threonine	612.7 $\pm$ 76	89	151.7 $\pm$ 25	66	461.0 $\pm$ 51	101
D-Threonine	649.8 $\pm$ 72	94	245.6 $\pm$ 34	107	404.1 $\pm$ 38	88
Inhibitor	Wild-type		<i>Abc-yaeE</i>		Wild-type- <i>Abc-yaeE</i>	
	Rate	%	Rate	%	Rate	%
—	287.8 $\pm$ 31	100	68.4 $\pm$ 11	100	219.3 $\pm$ 20	100
D-Methionine	34.2 $\pm$ 4	12	28.8 $\pm$ 5	42	5.4 $\pm$ 1	2
L-Methionine	66.7 $\pm$ 12	23	36.5 $\pm$ 5	53	31.1 $\pm$ 6	14
N-Formyl-L-methionine	71.6 $\pm$ 11	25	45.8 $\pm$ 8	67	25.73 $\pm$ 3	12
L-Alanine	273.0 $\pm$ 25	95	77.8 $\pm$ 9	114	195.2 $\pm$ 16	89
D-Alanine	306.5 $\pm$ 29	106	73.1 $\pm$ 7	107	233.33 $\pm$ 22	106
L-Leucine	269.1 $\pm$ 24	94	75.4 $\pm$ 14	110	193.7 $\pm$ 10	88
D-Leucine	258.2 $\pm$ 26	90	84.0 $\pm$ 4	123	174.2 $\pm$ 22	79
L-Valine	232.6 $\pm$ 21	81	65.3 $\pm$ 9	95	167.2 $\pm$ 12	76
L-Serine	327.5 $\pm$ 36	114	70.8 $\pm$ 10	103	256.7 $\pm$ 26	117
D-Serine	338.3 $\pm$ 23	118	85.6 $\pm$ 7	125	252.8 $\pm$ 17	115
L-Threonine	345.3 $\pm$ 33	120	84.0 $\pm$ 11	123	261.3 $\pm$ 22	119
D-Threonine	329.8 $\pm$ 33	115	87.9 $\pm$ 6	128	241.9 $\pm$ 27	110



inhibited by *N*-formyl L-methionine. However, no other amino acid inhibited strongly. Uptake by the *abc-yaeE* mutant was most strongly inhibited by L-methionine and to a lesser extent by L-threonine, L-valine, L-alanine, and L-leucine. When uptake of D-methionine was studied in wild-type *E. coli*, L-methionine was most inhibitory followed by *N*-formyl L-methionine and D-methionine in that order, but no other amino acid inhibited. In the *abc-yaeE* mutant, only L-methionine inhibited strongly. The mild increase of transport rate observed in the presence of some amino acids is unexplained. The results are consistent with the conclusion that the MetD transporter is specific for L- and D-methionine as well as *N*-formyl-L-methionine. The relative inhibitory effects of L- and D-methionine on uptake of these two radioactive substrates are in line with the relative affinities reported by Kadner (1974, 1977) ( $K_m$  values of 75 nM for L-methionine (Kadner 1974) and 1.2  $\mu$ M for D-methionine (Kadner 1977)). Inhibition studies of D-methionine uptake were also conducted with 100- and 1,000 fold excess of both L- and D-methionine. The results were consistent with the existence of a second low-affinity transporter for methionine (data not shown). Surprisingly, the uncharacterized methionine transporter present in the mutant is also fairly specific for methionine.

The effects of energy poisons were also examined (data not shown; see Fig. S3 in the electronic supplementary material). The conditions used were essentially the same as those reported in Zhang et al. (2003). Five mM sodium arsenate virtually abolished uptake of both L- and D-methionine under the same conditions used in the experiment reported in Fig. 2. FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) at a concentration of 2  $\mu$ M was substantially less inhibitory (see Fig. S3 in the electronic supplementary material). The results clearly suggest that uptake is energy dependent and are consistent with the expectation that ATP is the energy source.

#### Regulation of *metD* operon expression by MetJ

A *metJ* knockout mutant was constructed, and *metJ* was cloned into plasmid pBAD24 for complementation studies. Using [ $^{14}$ C]D-methionine as the uptake substrate, the effects of *metJ* expression plus and minus L- and D-methionine were studied (Fig. 3). In Fig. 3a, it can be seen that the presence of either L- or D-methionine substantially reduced uptake of D-methionine into wild-type cells. In Fig. 3b, the same experiment conducted with the *metJ* mutant revealed that (1) the loss of *metJ* enhanced uptake above that observed for the wild-type strain grown without methionine and (2) methionine present during growth did not exert a repressive effect. In Fig. 3c, it can be seen that the plasmid bearing *metJ* depressed methionine uptake activity in the wild-type background and depressed the much greater activity of the *metJ* mutant even more. As expected, the wild-type strain expressing *metJ* on the plasmid exhibited lower D-methionine uptake than observed for the *metJ* mutant bearing the same plasmid. These

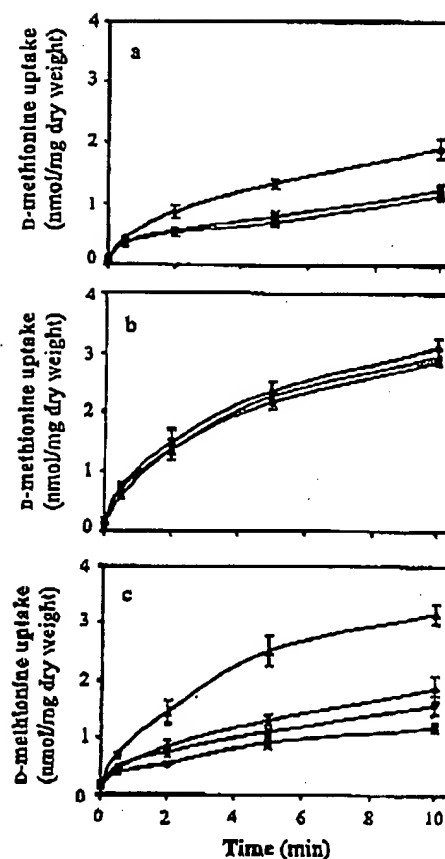


Fig. 3a–c Uptake of [ $^{14}$ C]D-methionine by cells grown in M9 medium with or without L- or D-methionine. Uptake experiments were conducted as described in the legend to Fig. 2. a Wild-type cells grown without methionine ( $\blacklozenge$ ), with 25  $\mu$ M L-methionine ( $\blacksquare$ ), or with 25  $\mu$ M D-methionine ( $\blacktriangle$ ). b The same conditions and symbols were used for the *metJ* mutant. c Complementation studies with the *metJ* bearing pBAD24 plasmid. Wild-type cells bearing pBAD24 ( $\blacklozenge$ ), wild-type cells bearing pBAD24-*metJ* ( $\blacksquare$ ), *metJ* mutant with pBAD24 ( $\blacktriangle$ ), *metJ* mutant with pBAD24-*metJ* ( $\bullet$ ). All cells in (c) were grown in medium M9 containing 100  $\mu$ g ampicillin ml $^{-1}$  and 2 mM L-arabinose.

results are in accord with the expected *metJ* gene dosages. Measurements of L-methionine uptake were similar, but differences were substantially less pronounced as expected (data not shown).

#### Phylogenetic studies

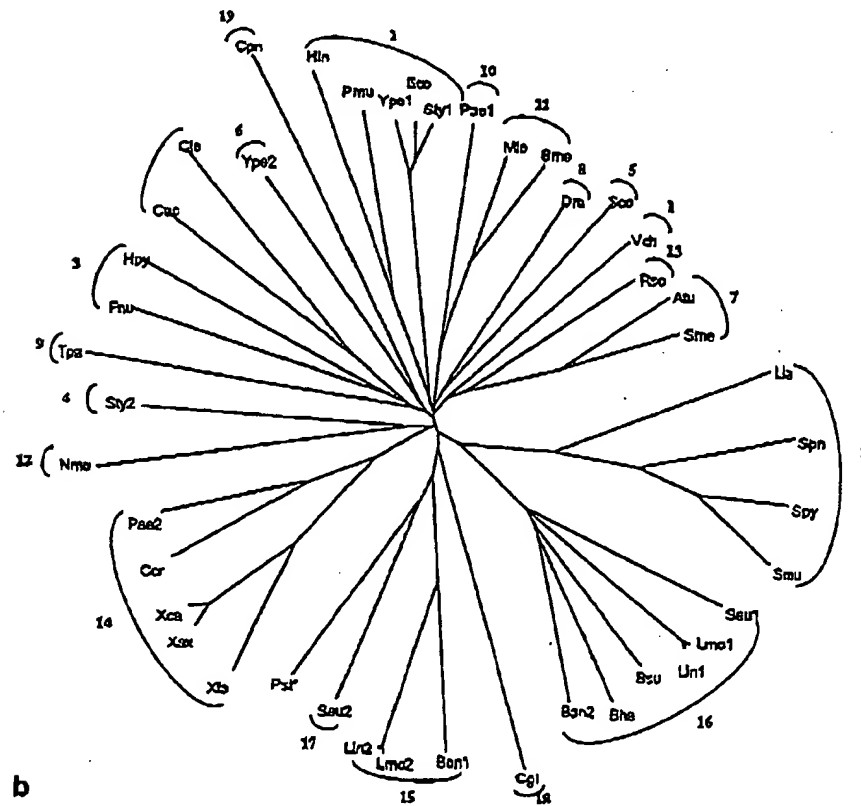
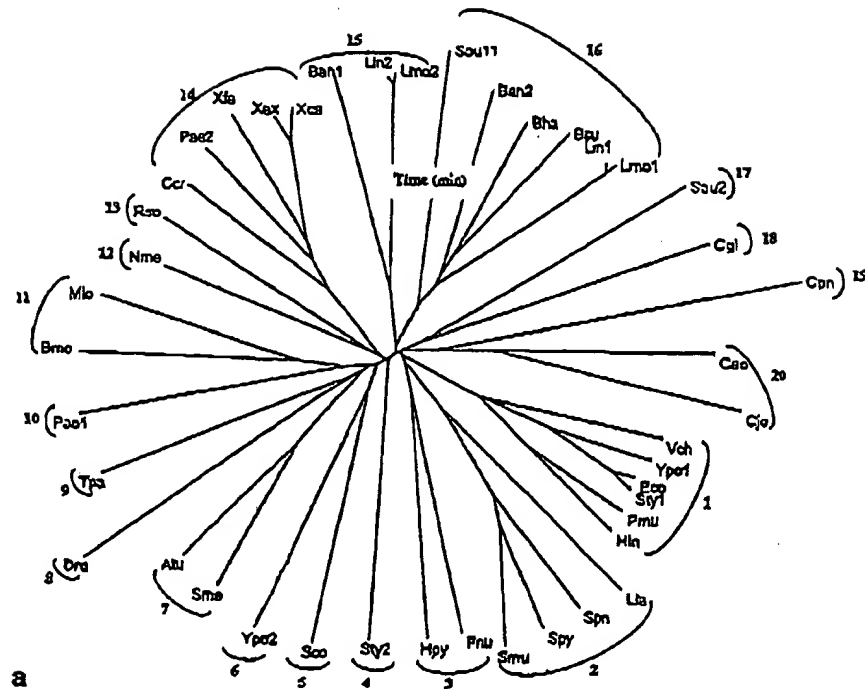
Our preliminary results suggested that the *E. coli* ABC methionine transporter identified by Gal et al. (2002) and Merlin et al. (2002) and in the work described here belongs to a novel family within the ABC superfamily. Surprisingly, this family is more closely related to the polar amino acid uptake transporter (PAAT) family (TC# 3.A.1.3) than to the hydrophobic amino acid uptake transporter (HAAT)

Phylogenetic cluster	Organism	Source	Protein abbreviation	ABC proteins		Membrane proteins		Substrate-binding receptors		
				Accession number	Size (aa)	Accession number	Size (aa)	Accession number	Size (aa)	Lipoprotein ?
1	<i>Salmonella typhimurium</i>	γ-Proteobacteria	Syl1	AAL19210	343	AAL19209	217	AAL19208	271	Y
	<i>Escherichia coli</i>	γ-Proteobacteria	Eco	AAC73310	343	AAC73309	217	AAC73308	271	Y
	<i>Yersinia pestis</i>	γ-Proteobacteria	Ype1	CAC89916	343	CAC89915	217	CAC89914	272	Y
	<i>Vibrio cholerae</i>	γ-Proteobacteria	Vch	AAF94069	344	AAF94068	225	AAF94067	275	Y
2	<i>Pasteurella multocida</i>	γ-Proteobacteria	Pmu	AAK03812	344	AAK03813	229	AAK03814	276	Y
	<i>Haemophilus influenzae</i>	γ-Proteobacteria	Hin	AAC22280	345	P46492	198	AAC22279	273	Y
	<i>Lactococcus lactis</i>	Firmicutes; Bacillales	Lla	CAB59828	368	AAK04421	231	CAB59827	286	N
	<i>Streptococcus pneumoniae</i>	Firmicutes; Bacillales	Spn	AAK98953	353	AAK98954	230	AAK98951	284	Y
3	<i>Streptococcus pyogenes</i>	Firmicutes; Bacillales	Spv	AAM78841	354	AAM78842	230	AAM78840	281	Y
	<i>Streptococcus mutans</i>	Firmicutes; Bacillales	Smu	AAL04079	354	AAL04080	229	AAL04077	280	Y
	<i>Escherichia coli</i>	Fusobacteria	Fnu	AAL94856	335	AAL94855	233	AAL94854	261	N
	<i>Helicobacter pylori</i>	α-Proteobacteria	Hpy	AAD08616	327	AAD08617	215	AAD08604	271	N
4	<i>Salmonella typhimurium</i>	γ-Proteobacteria	Sly2	AAL19465	338	AAL19466	219	AAL19464	276	N
	<i>Streptomyces coelicolor</i>	Firmicutes; Actinobacteria	Sco	CAB76078	368	CAB76077	240	CAB76076	275	Y
5	<i>Yersinia pestis</i>	γ-Proteobacteria	Ype2	CAC90148	328	CAC90149	223	CAC90147	274	N
	<i>Sinorhizobium meliloti</i>	α-Proteobacteria	Sme	CAC47469	358	CAC47468	221	CAC47467	258	N
6	<i>Agrobacterium tumefaciens</i>	α-Proteobacteria	Atu	AAL45281	346	AAK88954	222	AAK88953	259	N
	<i>Deinococcus radiodurans</i>	Thermus-Deinococcus group	Dra	AAF10928	325	AAF10929	218	AAF10931	256	N
7	<i>Treponema pallidum</i>	Spirochaetales	Tpa	AAC65110	269	AAC65109	219	AAF10930	256	N
	<i>Pseudomonas aeruginosa</i>	γ-Proteobacteria	Pae1	AAG05738	369	AAG05739	217	AAC65789	268	Y
8	<i>Brucella melitensis</i>	α-Proteobacteria	Bme	AAL53579	369	AAL53578	230	AAG07318	259	N
	<i>Mesorhizobium loti</i>	α-Proteobacteria	Mio	NP_105583	365	NP_105582	218	AAL53580	278	N
9	<i>Neisseria meningitidis</i>	β-Proteobacteria	Nme	CAB83797	245	CAB83798	228	AAL53135	268	N
	<i>Ralstonia solanacearum</i>	β-Proteobacteria	Rso	CAD14622	350	CAD14623	217	NP_105584	284	N
10								CAB83799	287	Y
								CAD14624	266	N

14	<i>Caulobacter crescentus</i>	α-Proteobacteria	Ccr	AAK24636	332	AAK24635	224	AAK24631	268	Y
	<i>Pseudomonas aeruginosa</i>	γ-Proteobacteria	Pac2	AAG08888	335	AAG08889	225	AAG08890	260	N
	<i>Xylella fastidiosa</i>	γ-Proteobacteria	Xfa	AAP83685	334	AAF83684	235	AAF83683	261	N
	<i>Xanthomonas axonopodis</i>	γ-Proteobacteria	Xax	AAM38512	335	AAM38511	231	AAM38510	269	N
	<i>Xanthomonas campestris</i>	γ-Proteobacteria	Xca	AAM42900	335	AAM42899	232	AAM42898	266	N
15	<i>Bacillus anthracis</i>	Firmicutes; Bacillales	Bao1	NP_654117	346	NP_654116	222	NP634118	270	Y
	<i>Listeria innocua</i>	Firmicutes; Bacillales	Lmo2	CAC95545	338	CAC95544	220	CAC95546	273	Y
	<i>Listeria monocytogenes</i>	Firmicutes; Bacillales	Lmo2	CAD008H	338	CAD00810	220	CAD00812	273	Y
16	<i>Staphylococcus aureus</i>	Firmicutes; Bacillales	Sau1	BAB56999	341	BAB57000	231	BAB57001	273	Y
	<i>Bacillus anthracis</i>	Firmicutes; Bacillales	Ban2	NP_653454	341	NP_653453	221	NP_653451	268	Y
	<i>Bacillus anthracis</i>	Firmicutes; Bacillales	Ban2	-	-	-	-	NP_J553452	270	Y
	<i>Bacillus halodurans</i>	Firmicutes; Bacillales	Bha	BAB07200	338	BAB07199	218	BAB07198	246	Y
	<i>Bacillus subtilis</i>	Firmicutes; Bacillales	Bsu	CAB 15264	341	CAB 15263	222	CAB 15262	274:	Y
	-	-	-	-	-	-	-	CAB 12739	263	Y
17	<i>Listeria innocua</i>	Firmicutes; Bacillales	Lin1	CAC97741	340	CAC97740	224	CAC97739	276	Y
	<i>Listeria monocytogenes</i>	Firmicutes; Bacillales	Lmo1	CAD00497	340	CAD00496	224	CAD00495	276	Y
18	<i>Staphylococcus aureus</i>	Firmicutes; Bacillales	Sau2	BAB56624	341	BAB56625	219	BAB56626	280	Y
	<i>Corynebacterium glutamicum</i>	Firmicutes; Actinobacteria	Cgl	NP599870	360	NP599869	225	NP599871	299	Y
19	<i>Chlamydia pneumoniae</i>	Chlamydiales	Cpn	AAD 18429	341	AAD18428	221	AAD 18427	272	N
20	<i>Clostridium acetobutylicum</i>	Firmicutes; Bacillus-Clostridium group	Cac	AAK78960	320	AAK78961	218	AAK78962	272	Y
	<i>Campylobacter jejuni</i>	α-proteobacteria	Cje	CAB73039	336	CAB73038	303	CAB73037	257	N
	-	-	-	-	-	-	-	CAB73036	256	N
	-	-	-	-	-	-	-	CAB73035	258	N
	-	-	-	-	-	-	-	-	-	-
	<i>Providencia stuartii</i> *	γ-Proteobacteria	Psi	-	-	AAF71397	146	AAA25538	277	Y
	<i>Mannheimia haemolytica</i> *	γ-Proteobacteria	Mha1	-	-	-	-	AAA25547	277	Y
	-	-	Mha2	-	-	-	-	AAA25540	263	Y
	-	-	Mha3	-	-	-	-	CAA06664	259	Y
	<i>Legionella pneumophila</i> *	γ-Proteobacteria	Lpn	-	-	-	-	-	-	-
	<i>Neisseria gonorrhoeae</i> *	β-Proteobacteria	Ngo	-	-	-	-	AAF4768	288	Y

96

Fig. 4a-c Phylogenetic trees of the three constituents of the MUT family of ABC transporters: a ATP-binding cassette (ABC) constituents, b membrane constituents, c solute binding receptors. The multiple alignments were generated with the ClustalX program (Thompson et al. 1997) using the BLOSUM 62 scoring matrix. The trees are based on the neighbor-joining method and were drawn with the Tree-View program (Page 1996). c\* refers to a receptor that is not encoded with a gene for an ABC protein or a membrane protein



19 (Cpn  
14 (Xba  
Xba\*  
Xba  
Cor  
10 (Pae1  
Pae2  
Dra1  
Dra2\*  
8 (Ypa2  
6 (Sac  
3 (Fru  
Hpy  
20 (Ceo  
Cfo1  
Cfo2\*  
Cfo3\*  
9 (Tpa  
11a  
Spm  
Spy  
Smu  
Bsu2\*  
Sau2  
Cgl  
13 (Bmo1  
Bmo2\*  
11 (Mio  
7 (Smo  
Alu  
12 (Nga\*  
Nma  
Sse2  
4  
15 (Lna2  
Lna1  
Bna1  
1 (Mha2  
Mha1  
Hln  
Eco2\*  
Lpn\*  
Pmu  
Mha3  
Vch  
Ypa1  
Eco1  
Sly1  
16 (Bsu1  
Ban2  
Lna1  
Lmo1  
Bra  
1 (Cfo1  
Cfo2\*  
Cfo3\*  
Tpa  
11a  
Spm  
Spy  
Smu  
Bsu2\*  
Sau2  
Cgl  
13 (Bmo1  
Bmo2\*  
11 (Mio  
7 (Smo  
Alu  
12 (Nga\*  
Nma  
Sse2  
4

Cluster 3 proteins from *Helicobacter pylori* and *Fusobacterium nucleatum* always cluster together in spite of the great phylogenetic distance between these two organisms. It seems possible that horizontal transfer had occurred. Therefore, the G+C contents and codon usages of the genes encoding the ABC transporters of these two organisms were compared, but no significant differences were observed relative to those of the genomes. This negative result does not eliminate the possibility of horizontal transfer.

98

a	Eco	AGACGTCT	GGATGCCT	TAACATCC	-67
	Styl	AGACGTCT	GGATGCCT	TAACATCC	-68
	Ypel	AGCCGTCT	AGACGCCT	TAACATCC	-64
	Vch	AGACGTCT	AGACGTAA	AAATATCT	-92
	Hin	AGCAATCT	AGACATCT		-39
b	Pmu	GGAAATCT	AGACGTCT		-40
	Eco	8/8	5/8	4/8	
	Styl	8/8	5/8	4/8	
	Ypel	7/8	7/8	4/8	
	Vch	8/8	6/8	5/8	
	Hin	5/8	7/8		
	Pmu	5/8	8/8		

Fig. 5a, b Conserved MetJ binding sites (Met boxes) found between *abc* and *yaeD* from enterobacteria. a An alignment of proposed Met boxes from *Escherichia coli* (Eco), *Salmonella typhimurium* (Styl), *Yersinia pestis* (Ypel), *Vibrio cholerae* (Vch), *Haemophilus influenzae* (Hin), and *Pasteurella multocida* (Pmu). Protein abbreviations are as indicated in Table 4. The numbers at the right indicate the distance from the start codon of *abc*. b The number of matches to the consensus Met box sequence (AGACGTCT) is shown for each predicted Met box

Clusters 4–13 consist of only one or two proteins per cluster. In clusters 7 and 11, the two proteins in each cluster are from  $\alpha$ -Proteobacteria, suggesting orthology. However, some of the distantly related proteins belong to closely related organisms (e.g., Sty2 and Ype2; Nme and Rso). This clearly suggests that sequence-divergent primordial proteins resulted from early gene duplication events and that these early paralogues were not transmitted to most of the organisms. Moreover, this conclusion is confirmed by the presence of several lipoproteic receptors from gram-negative organisms (Ccr, Nme, Tpa) although most are soluble (Table 4).

Cluster 14 consists of  $\gamma$ -proteobacterial proteins except for Ccr, which is from an  $\alpha$ -proteobacterial species. The clustering patterns in Fig. 4a, c are consistent with orthology, but the clustering of Ccr in Fig. 4b is anomalous, and the same topology was obtained using PAM distances as a model of evolution.

Clusters 15 and 16 clearly represent two sequence-divergent groups of paralogues, both represented in *Bacillus anthracis* and two *Listeria* species. *Staphylococcus aureus* as well as *Bacillus subtilis* and *Bacillus halodurans* encode within their genomes only the second of these homologues (cluster 16). The clustering of Bha in Fig. 4c is anomalous, and this was also observed for the tree drawn using PAM distances. S-boxes have been predicted upstream of the operons encoding all transporters in clusters 15 and 16 with the exception of Bha (data not shown).

Cluster 19 includes a single chlamydial protein although three chlamydial species have been sequenced. In spite of their close phylogenetic relationship, the other two close relatives of *Chlamydia pneumoniae* must have lost the corresponding orthologues.

Finally, in cluster 20, Cac and Cjc are always together (Fig. 4a, b, c). Because of the great distance between

*Clostridium acetobutylicum* (a gram-positive bacterium) and *Campylobacter jejuni* (a gram-negative  $\epsilon$ -proteobacterium), we suggest that horizontal transfer has occurred. However, this assumption can not be confirmed as both organisms have similar overall G+C content, and the G+C content and codon usage patterns of the MUT genes did not prove to be different from those of the complete genomes. An S-box was also predicted in the region of the operon encoding the Cac system.

With the exception of Lia, all receptors from gram-positive organisms were predicted to be lipoproteins. For Lia, a signal peptide with high similarity to those of Spn, Spy, and Smu was found. However, the conserved cysteine residue essential for lipid anchorage was replaced by a glycine residue. This might be explained by a sequencing error or by a single-point mutation since the codons for these two residues differ by only one nucleotide.

## Discussion

The results presented here confirm and extend the molecular characterization of the MetD transporter identified earlier in the laboratories of Kadner and Ayling, *abc* and *yaeE* encode, respectively, the ATP-binding cassette (ABC) and membrane proteins of the MetD transporter. YaeC, the receptor encoded with the other components of the transporter, is the major binding protein for both L- and D-methionine. Inhibition studies revealed that the transporter is specific for both methionine isomers and their analogues including N-formyl methionine (Table 3; Kadner 1974, 1977). However, the YaeC-related paralogue NlpA (lipoprotein 28) may also exhibit the slight capacity to bind the two isomers of methionine. The *abc-yaeE-yaeC* genes were renamed *metNIQ* by Gal et al. (2002) and Merlin et al. (2002).

We confirmed Kadner's observation that L-methionine effectively competes for D-methionine transport while D-methionine does not strongly compete for L-methionine transport. This led Kadner to suggest that the *metD* locus encodes a component of at least two transport systems but may not encode the initial methionine-binding site (Kadner 1977), a suggestion reiterated by Merlin et al. (2002). However, the data presented here show that MetD is a single transport system with a single major methionine-binding receptor. The difference in inhibition observed for the two isomers is a consequence of the low  $K_m$  for L-methionine (75 nM) (Kadner 1974), which is 15-fold lower than that for D-methionine (1.2  $\mu$ M) (Kadner 1977). We further demonstrate that MetJ is an effective repressor of *metD* expression.

Phylogenetic analyses led to the conclusion that MetD belongs to a new ABC family, which we named the methionine uptake transporter (MUT) family (TC #3.A.1.23). The MUT family is widely represented among bacterial subdivisions. All homologues of each of the three components are of a similar size, and all membrane proteins exhibit five putative transmembrane  $\alpha$ -helical segments (TMSs). The overall topology of the trees presented does not fol-

low the phylogenies of the organisms, suggesting that several genetic duplications encoding these systems had occurred early during the evolutionary history of the family. The existence of several sequence-divergent primordial paralogues is likely to explain the topology of the phylogenetic trees. However, no more than two of these paralogues have been transmitted to any currently sequenced organism.

It is interesting to note that *Yersinia pestis* and *Salmonella typhimurium*, two close relatives of *E. coli*, possess two paralogous systems within the MUT family whereas only the receptor is present twice in *E. coli*. The possibility that the common ancestor of these three organisms had two paralogous systems, and that *E. coli* specifically lost the membrane and ABC proteins of one of them is highly unlikely. Ype2 and Sty2 do not cluster with Ype1 and Sty1 although the two receptors, Eco2 and Eco1, are found together in cluster 1. We therefore propose that *nlpA* (encoding Eco2) arose by a much more recent duplication of the *yacC* gene precursor (encoding Eco1). This conclusion is corroborated by the observation that both Sty2 and Ype2 are not lipoproteins although Eco1 and Eco2 are.

Cluster 1 includes a group of  $\gamma$ -proteobacterial proteins conserved in all three trees. For this cluster, the phylogenies of the proteins agree with those of the organisms. Moreover, we identified MetJ binding sites in the promoter regions of all members of this cluster, and all cluster 1 receptors are predicted to be lipoproteins. These observations strongly suggest that all cluster 1 systems are orthologous methionine transporters, a suggestion confirmed by the presence of the functionally similar MetD transporter in *S. typhimurium* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Several members of the MUT family from gram-positive bacteria are likely to transport sulfur compounds. Thus, almost all cluster 15 and 16 constituents (Figs. 4a-c) are encoded in operons regulated by S-boxes (Grundy and Henkin 1998). S-boxes are gram-positive bacterial consensus sequences for the transcriptional control of sulfur metabolism (Grundy and Henkin 1998). The presence of transporters likely to be involved in sulfur acquisition in positions throughout the phylogenetic tree suggests that this entire family may be involved in the transport of organic sulfur compounds. Further experimentation will be required to determine the substrate ranges of the transporters in this family.

The MUT family is of pharmaceutical interest since several members are required for bacterial pathogenicity. *sfhA* of *S. typhimurium* is found in a pathogenicity islet and is essential for infection in a mouse model (Pattery et al. 1999), although its specific contribution to pathogenicity is unknown. *sfhA* was predicted to encode the binding protein of an ABC transporter for iron because its expression was increased under iron-limiting conditions. Despite its regulation by iron, a Fur regulatory binding site was not found close to this operon (Panina et al. 2001). Based on the analyses presented here, we suggest that the Sfb transporter is involved in the uptake of an amino acid or

sulfur compound during infection. The *H. influenzae hlpA* gene is not essential for infection, but a mutation in this gene results in reduced invasion in rats (Chanyangam et al. 1991). *Helicobacter pylori* also contains a MUT family transporter (AbcBCD) of unknown function that is required for maximal production of urease, essential for colonization (Hendricks and Mobley 1997). Since many *Yersinia pestis* strains require exogenous methionine (Brubaker 1972), methionine transporters may be drug targets for these organisms.

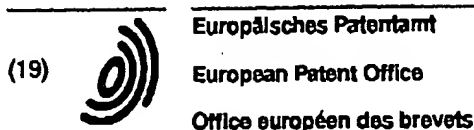
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**EP 1 038 970 A2**

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**(54) Method for producing L-glutamic acid**

(57) L-Glutamic acid is more efficiently produced at lower cost compared with conventional techniques by culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.

EP 1 038 970 A2

## EP 1 038 970 A2

## Description

## Technical Field

5 [0001] The present invention relates to an L-glutamic acid-producing bacterium and a method for producing L-glutamic acid by fermentation utilizing the bacterium. L-glutamic acid is an amino acid important for foodstuffs, medical supplies and so forth.

## Related Art

10 [0002] Heretofore, L-glutamic acid has been produced by a fermentation method using coryneform L-glutamic acid-producing bacteria belonging to the genus *Brevibacterium*, or *Corynebacterium* (Amino Acid Fermentation, Gakkoai Shuppan Center, pp.195-215, 1986). As such coryneform bacteria, wild strains isolated from nature or mutants thereof are used to improve the productivity.

15 [0003] Moreover, there have also been disclosed various techniques for promoting L-glutamic acid-producing ability by enhancing genes for enzymes involved in the L-glutamic acid biosynthetic system through recombinant DNA techniques. For example, Japanese Patent Laid-open Publication No. 63-214189 discloses a technique for elevating the L-glutamic acid-producing ability by enhancing a glutamate dehydrogenase gene, isocitrate dehydrogenase gene, aconitate hydratase gene, and citrate synthase gene.

20 [0004] On the other hand, as for L-threonine, there has been known a technique of disruption of an uptake system of the amino acid in order to increase the production of the amino acid (Okamoto, K. et al., *Biosci. Biotech. Biochem.*, 61 (11), 1877-1882 (1997)).

[0005] For L-glutamic acid, the structure of the gene cluster of the uptake system for L-glutamic acid (*gluABCD* operon) in *Corynebacterium glutamicum* has been known (Kronmeyer, W. et al., *J. Bacteriol.*, 177 (5), 1152-1158 (1995)). Moreover, Kronmeyer et al. produced a strain in which the L-glutamic acid uptake system encoded by the *gluABCD* operon was deleted, and studied about the excretion of L-glutamic acid using this strain. In this study, they concluded that the excretion of L-glutamic acid of *Corynebacterium glutamicum* did not depend on *gluABCD*, and depended on other uptake and excretion mechanisms. Furthermore, an uptake system of L-glutamic acid other than that encoded by *gluABCD* has also been reported (Burkovski, A. et al., *FEMS Microbiology Letters*, 136, 169-173 (1996)). These reports suggest that the accumulation amount of L-glutamic acid in the medium is not affected even if at least the L-glutamic acid uptake system encoded by the *gluABCD* operon is deleted. Therefore, it has not been attempted to improve the L-glutamic acid-producing ability by disruption of the uptake system of L-glutamic acid encoded by *gluABCD*.

## 35 DETAILED DESCRIPTION OF THE INVENTION

[0006] The object of the present invention is to breed a bacterial strain having high L-glutamic acid-producing ability, and thereby provide a method for more efficiently producing L-glutamic acid at low cost in order to respond to further increase of the demand of L-glutamic acid.

40 [0007] In order to achieve the aforementioned object, the inventors of the present invention studied assiduously. As a result, they found that an L-glutamic acid-producing bacterium of coryneform bacteria whose *gluABCD* operon was deleted had high L-glutamic acid-producing ability contrary to the suggestion by the aforementioned prior art, and thus accomplished the present invention.

[0008] That is, the present invention provides the followings.

- 45 (1) A method for producing L-glutamic acid, comprising the steps of culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.
- 50 (2) The method according to (1), wherein the L-glutamic acid uptake system is encoded by the *gluABCD* operon.
- (3) The method according to (2), wherein at least one of expression products of the *gluABCD* operon is deleted in the coryneform bacterium.
- (4) The method according to (3), wherein at least *gluA* is deleted in the coryneform bacterium.
- (5) The method according to (4), wherein all of *gluA*, *gluB*, *gluC* and *gluD* are deleted in the coryneform bacterium.

55 [0009] According to the present invention, L-glutamic acid can be produced at a higher yield compared with conventional techniques.

[0010] For the present invention, the term "L-glutamic acid-producing ability" refers to an ability of a coryneform

## EP 1 038 970 A2

bacterium used for the present invention to accumulate L-glutamic acid in a medium when the bacterium is cultured in the medium.

[0011] Hereafter, the present invention will be explained in detail.

[0012] The coryneform bacterium used for the present invention is a coryneform bacterium having L-glutamic acid-producing ability, in which an L-glutamic acid uptake system is deleted or decreased.

[0013] Bacteria belonging to the genus *Corynebacterium* as referred to herein are a group of microorganisms defined in Bergey's Manual of Determinative Bacteriology, 8th Ed., p. 599 (1974). The bacteria are aerobic, Gram-positive, nonacid-fast bacilli not having the ability to sporulate, and include bacteria which had been classified as bacteria belonging to the genus *Brevibacterium* but have now been unified into the genus *Corynebacterium* (see Int. J. Syst. Bacteriol., 41, 255 (1981)) and also include bacteria of the genus *Brevibacterium* and *Microbacterium* which are closely related to the genus *Corynebacterium*. Examples of coryneform bacteria preferably used for producing L-glutamic acid include the followings.

*Corynebacterium acetoacidophilum*  
*Corynebacterium acetoglutamicum*  
*Corynebacterium alkanolyticum*  
*Corynebacterium callunae*  
*Corynebacterium glutamicum*  
*Corynebacterium lilium* (*Corynebacterium glutamicum*)  
*Corynebacterium melassecola*  
*Corynebacterium thermoaminogenes*  
*Corynebacterium hercullis*  
*Brevibacterium divaricatum* (*Corynebacterium glutamicum*)  
*Brevibacterium flavum* (*Corynebacterium glutamicum*)  
*Brevibacterium immariophilum*  
*Brevibacterium lactofermentum* (*Corynebacterium glutamicum*)  
*Brevibacterium roseum*  
*Brevibacterium saccharolyticum*  
*Brevibacterium thiogenitalis*  
*Brevibacterium ammoniagenes* (*Corynebacterium ammoniagenes*)  
*Brevibacterium album*  
*Brevibacterium cerinum*  
*Microbacterium ammoniaphilum*

[0014] Specifically, the following strains of these bacteria are exemplified:

*Corynebacterium acetoacidophilum* ATCC13870  
*Corynebacterium acetoglutamicum* ATCC15806  
*Corynebacterium alkanolyticum* ATCC21511  
*Corynebacterium callunae* ATCC15991  
*Corynebacterium glutamicum* ATCC13020, 13032, 13060  
*Corynebacterium lilium* (*Corynebacterium glutamicum*) ATCC15990  
*Corynebacterium melassecola* ATCC17965  
*Corynebacterium thermoaminogenes* AJ12340 FERN BP-1539)  
*Corynebacterium hercullis* ATCC13868  
*Brevibacterium divaricatum* (*Corynebacterium glutamicum*) ATCC14020  
*Brevibacterium flavum* (*Corynebacterium glutamicum*) ATCC13826, ATCC14067  
*Brevibacterium immariophilum* ATCC14068  
*Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) ATCC13665, ATCC13869  
*Brevibacterium roseum* ATCC13825  
*Brevibacterium saccharolyticum* ATCC14066  
*Brevibacterium thiogenitalis* ATCC19240  
*Corynebacterium ammoniagenes* (*Brevibacterium ammoniagenes*) ATCC6871  
*Brevibacterium album* ATCC15111  
*Brevibacterium cerinum* ATCC15112  
*Microbacterium ammoniaphilum* ATCC15354

[0015] The aforementioned deletion or decrease of an L-glutamic acid uptake system of coryneform bacteria can

## EP 1 038 970 A2

be attained by mutating or disrupting a gene coding for the uptake system using a mutagenesis treatment or a genetic recombination technique. The term "L-glutamic acid uptake system is deleted or decreased" means to make the uptake system not function normally, and it includes that at least one of proteins constituting the uptake system is deleted or the activity of the protein is decreased, and that two or more of the proteins are deleted or the activities of the proteins are decreased. Further, the term "a protein is deleted" used herein include both of a case where the protein is not expressed at all, and a case where a protein that does not normally function is expressed.

[0016] The gene coding for the L-glutamic acid uptake system can be disrupted by gene substitution utilizing homologous recombination. A gene on a chromosome of a coryneform bacterium can be disrupted by transforming the coryneform bacterium with DNA containing a gene modified by deleting its internal sequence (deletion type gene) so that the uptake system should not function normally to cause recombination between the deletion type gene and a normal gene on the chromosome. Such gene disruption utilizing homologous recombination has already been established, and there have been known methods therefor utilizing linear DNA, plasmid containing a temperature sensitive replication origin and so forth. A method utilizing a plasmid containing a temperature sensitive replication origin is preferred.

[0017] As a gene coding for an L-glutamic acid uptake system, the *gluABCD* operon has been known (Kronmeyer, W. et al., *J. Bacteriol.*, 177 (5), 1152-1158 (1995)). Moreover, an L-glutamic acid uptake system other than that encoded by the *gluABCD* operon has also been known (Burkovski, A. et al., *FEMS Microbiology Letters*, 136, 169-173 (1996)). Although any of such genes may be disrupted, it is preferred that the uptake system encoded by the *gluABCD* operon is disrupted.

[0018] Because the nucleotide sequence of this operon has been known (GenBank/EMBL/DDBJ Accession X81191), this operon or each structural gene in the *gluABCD* operon can be isolated from chromosome DNA of coryneform bacteria by PCR using primers produced based on the nucleotide sequence. A certain region can be excised from the thus obtained gene fragment with one or more restriction enzymes, and at least a part of a coding region or an expression control sequence such as promoter can be deleted to prepare a deletion type gene.

[0019] Further, a deletion type gene can also be obtained by performing PCR using primers designed so that a part of a gene should be deleted. For example, by using the primers having the nucleotide sequences shown as SEQ ID NOS: 1 and 2 in Sequence Listing, a *gluD* gene having a deletion of a part of 5' sequence can be obtained. Further, by using the primers having the nucleotide sequences shown as SEQ ID NOS: 3 and 4, a *gluA* gene having a deletion of a part of 3' sequence can be obtained. When gene substitution is performed by using the deletion type *gluA* gene or *gluD* gene obtained by using those primers, the *gluA* gene or the *gluD* gene can be disrupted. Further, if these deletion type *gluA* gene and deletion type *gluD* gene are ligated, and the obtained fusion gene is used for gene substitution, all of *gluA*, *gluB*, *gluC* and *gluD* can be disrupted. Furthermore, when PCR is performed by using a plasmid containing a *gluA* gene that has been obtained by using primers having the nucleotide sequences shown as SEQ ID NOS: 3 and 5 as a template, and primers having the nucleotide sequences shown as SEQ ID NOS: 6 and 7, and the amplification product is cyclized, there can be obtained a plasmid containing *gluA* gene including a deletion of an internal sequence and having 5' region and 3' region ligated in-frame. When gene substitution is performed by using this plasmid, only the *gluA* gene can be disrupted.

[0020] Moreover, primers other than those exemplified above can also be designed by the methods well known to those skilled in the art, and an arbitrary structural gene in the *gluABCD* operon can be disrupted by using such primers. Alternatively, the uptake system can be deleted by deleting an expression control sequence of the *gluABCD* operon such as a promoter so that the gene cannot be expressed.

[0021] While the gene substitution of the *gluABCD* gene-cluster (henceforth referred to simply as "*gluABCD* gene") will be explained below, an arbitrary structural gene or expression control sequence can be similarly deleted.

[0022] The *gluABCD* gene on a host chromosome can be replaced with a deletion type *gluABCD* gene as follows. That is, a recombinant DNA is constructed by insertion of a temperature sensitive replication origin, a deletion type *gluABCD* gene and a marker gene expressing resistance to a drug such as chloramphenicol, tetracycline and streptomycin, and a coryneform bacterium is transformed with this recombinant DNA. Then, the transformant strain can be cultured at a temperature at which the temperature sensitive replication origin does not function, and then cultured in a medium containing a corresponding drug to obtain a transformant strain in which the recombinant DNA is integrated into chromosomal DNA.

[0023] In such a strain in which a recombinant DNA is integrated into a chromosome as described above, recombination of the *gluABCD* gene sequence originally present on the chromosome has been caused, and two fusion genes of the chromosomal *gluABCD* gene and the deletion type *gluABCD* gene are inserted into the chromosome, between which the other parts of the recombinant DNA (the vector portion, temperature sensitive replication origin, and drug resistance marker) are present. Therefore, because the normal *gluABCD* gene is dominant in this state, the transformant strain expresses the L-glutamic acid uptake system.

[0024] Then, in order to leave only the deletion type *gluABCD* gene on the chromosome DNA, one copy of the *gluABCD* gene is eliminated from the chromosome DNA together with the vector portion (including temperature sensitive replication origin and drug resistance marker) by recombination of the two *gluABCD* gene. Upon the recombination,

## EP 1 038 970 A2

the normal *gluABCD* gene may be left on the chromosome DNA and the deletion type *gluABCD* gene may be excised, or the deletion type *gluABCD* gene may be left on the chromosome DNA and the normal *gluABCD* gene may be excised. In both of the cases, the excised DNA is retained on the plasmid in a cell when the cell is cultured at a temperature at which the temperature sensitive replication origin functions. Such DNA on the plasmid is eliminated from the cell when the cell is cultured at a temperature at which the temperature sensitive replication origin does not function. It can be confirmed which genes are left on the chromosome DNA by investigating the structure of the *gluABCD* gene on the chromosome by PCR, hybridization or the like.

[0025] When such a *gluABCD* gene-disrupted strain produced as described above is cultured at a temperature at which the temperature sensitive replication origin functions (for example, low temperature), the *gluABCD* gene will be retained in its cell. When it is cultured at a temperature at which the temperature sensitive replication origin does not function (for example, elevated temperature), the *gluABCD* gene will be eliminated from the cell.

[0026] Examples of the plasmid which has a temperature sensitive replication origin functioning in coryneform bacterial cells include, pHS4, pHSC4, pHS22, pHSC22, pHS23, pHSC23 (as for these, see Japanese Patent Publication (Kokoku) No. 7-108228) and so forth. These temperature sensitive plasmids can autonomously replicate at a temperature of about 10-32°C, but cannot autonomously replicate at a temperature of about 34°C or higher in a coryneform bacterial cell.

[0027] After the *gluABCD* gene on the chromosome are disrupted as described above, the genes-disrupted strain is preferably introduced with *recA*<sup>+</sup> because such introduction of *recA*<sup>+</sup> prevents the *gluABCD* gene on the plasmid from being integrated again into the chromosome again during culture at a low temperature.

[0028] The coryneform bacterium used for the present invention may have enhanced activity of an enzyme for catalyzing the biosynthesis of L-glutamic acid in addition to the deletion or decrease of L-glutamic acid uptake system. Illustrative examples of the enzyme for catalyzing the biosynthesis of L-glutamic acid include glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose biphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and the like.

[0029] Further, in the coryneform bacterium used for the present invention, an enzyme that catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid may be decreased or deleted. Illustrative examples of the enzyme which catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid include a-ketoglutarate dehydrogenase, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetylhydroxamate synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and the like.

[0030] Furthermore, by introducing a thermosensitive mutation for a biotin activity inhibiting substance such as surface active agents into a coryneform bacterium having L-glutamic acid-producing ability, the bacterium becomes to be able to produce L-glutamic acid in a medium containing an excessive amount of biotin in the absence of a biotin activity inhibiting substance (see WO96/06180). As such a coryneform bacterium, the *Brevibacterium lactofermentum* AJ13029 strain disclosed in WO96/06180 can be mentioned. The AJ13029 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology on September 2, 1994, and received an accession number of FERM P-14501. Then, it was transferred to an international deposition under the provisions of the Budapest Treaty on August 1, 1995, and received an accession number of FERM BP-5189.

[0031] When a coryneform bacterium having L-glutamic acid-producing ability, in which the L-glutamic acid uptake system is deleted or decreased, is cultured in a suitable medium, L-glutamic acid is accumulated in the medium. Because of the deletion or decrease of L-glutamic acid uptake system in the coryneform bacterium used for the present invention, L-glutamic acid secreted from the cell is prevented from being taken up again into the cell. As a result, the accumulation amount of L-glutamic acid in the medium is increased. According to the method of the present invention, improvement of interval yield (ratio of the accumulation amount of L-glutamic acid to the consumption of saccharides in a certain period of cultivation) can be expected when L-glutamic acid concentration in the medium becomes high. In particular, when a highly productive strain that shows a high L-glutamic acid concentration in a medium during fermentation is used, a marked effect can be obtained.

[0032] The medium used for producing L-glutamic acid by utilizing the microorganism of the present invention is a usual medium that contains a carbon source, a nitrogen source, inorganic ions and other organic trace nutrients as required. As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, starch hydrolysate and molasses or the like; alcohols such as ethanol, inositol; or organic acids such as acetic acid, fumaric acid, citric acid and succinic acid or the like.

[0033] As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate and ammonium acetate, ammonia, organic nitrogen such as peptone, meat extract, yeast extract, corn steep liquor and soybean hydrolysates, ammonia gas, aqueous ammonia

## EP 1 038 970 A2

and so forth.

[0034] As the inorganic ions (or sources thereof), added is a small amount of potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth. As for the organic trace nutrients, it is desirable to add required substances such as vitamin B1, yeast extract and so forth in a suitable amount as required.

6 [0035] The culture is preferably performed under an aerobic condition attained by shaking, stirring for aeration or the like for 16 to 72 hours. The culture temperature is controlled to be at 30°C to 45°C, and pH is controlled to be 5 to 9 during the culture. For such adjustment of pH, inorganic or organic acidic or alkaline substances, ammonia gas and so forth can be used.

10 [0036] Collection of L-glutamic acid from fermentation broth can be attained by, for example, methods utilizing ion exchange resin, crystallization and so forth. Specifically, L-glutamic acid can be adsorbed or isolated by an anion exchange resin, or crystallized by neutralization.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 [0037]

Fig. 1 shows the scheme of construction of a plasmid pTΔAD for disrupting *gluABCD* gene.

Fig. 2 shows the scheme of construction of a plasmid pTΔA for disruption of *gluA*.

#### 20 BEST MODE FOR CARRYING OUT THE INVENTION

[0038] The present invention will be further specifically explained hereinafter with reference to the following examples.

##### 25 (1) Construction of plasmid for disruption of *gluABCD* gene

[0039] In order to create a *gluABCD* gene-disrupted strain of coryneform bacterium by homologous recombination using a temperature sensitive plasmid, a plasmid for disruption of the *gluABCD* gene was constructed.

30 [0040] First, a deletion type *gluABCD* gene was constructed by cloning a *gluD* gene having a deletion of 5' sequence, and ligating it with a *gluA* gene having a deletion of 3' sequence. Specifically, a fragment of about 300 bp from a *Bam*HI site present in *gluD* to a site about 270 bp downstream from *gluD* was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869, which was a wild-type strain of coryneform bacterium, by PCR utilizing oligonucleotides having the nucleotide sequences represented as SEQ ID NOS: 1 and 2 as primers. This amplified fragment was digested with *Bam*HI and *Xba*I, and the obtained fragment was ligated to pHSΔ299 (produced by Takara Shuzo) digested with *Bam*HI and *Xba*I using T4 ligase (produced by Takara Shuzo) to obtain a plasmid pHSΔ*gluD*.

35 [0041] Then, a fragment of about 300 bp from a site about 180 bp upstream from *gluA* to the *Bam*HI site present in *gluA* was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by PCR utilizing oligonucleotides having the nucleotide sequences represented as SEQ ID NOS: 3 and 4 as primers. This amplified fragment was digested with *Eco*RI and *Bam*HI, and the obtained fragment was ligated to pHSΔ*gluD* digested with *Eco*RI and *Bam*HI by using T4 ligase to obtain a plasmid pHSΔ*gluAD*. This plasmid had a structure in which *gluB* and *gluC* were deleted, and parts of *gluA* and *gluD* were ligated.

40 [0042] Then, in order to make pHSΔ*gluAD* autonomously replicable in coryneform bacteria, a temperature sensitive replication origin derived from a plasmid autonomously replicable in coryneform bacteria was introduced into the unique *Hinc*II cleavage site in pHSΔ*gluAD*. Specifically, the following procedure was used.

45 [0043] A plasmid pHSC4 containing a temperature sensitive replication origin (see Japanese Patent Laid-open Publication (Kokai) No. 5-7491) was digested with *Bam*HI and *Kpn*I. The both termini of the obtained DNA fragment was blunt-ended using Blunting Kit (produced by Takara Shuzo), ligated with a *Kpn*I linker (produced by Takara Shuzo), and then allowed to cause self-ligation to obtain pKCT4. pHSC4 was a plasmid obtained as follows. That is, a DNA fragment containing a replication origin was excised from a plasmid pAJ1844 (see Japanese Patent Laid-open Publication (Kokai) No. 58-216199), which had a replication origin derived from pHM1519 (K. Miwa et al., *Agric. Biol. Chem.*, 48, 2901-2903 (1984), Japanese Patent Laid-open Publication (Kokai) No. 58-77895), and ligated to a plasmid for *Escherichia coli*, pHSΔ298, to obtain a shuttle vector pHK4. This pHK4 was treated with hydroxylamine to obtain a plasmid pHS4 modified to be temperature sensitive. The temperature sensitive replication origin was excised from pHS4, and ligated to pHSΔ398 to obtain pHSC4. *Escherichia coli* AJ12571 harboring pHSC4 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology on October 11, 1990, and received the accession number of FERM P-11763. Then, it was transferred to international deposit under the Budapest Treaty on August 26, 1991, and received the accession number of FERM BP-3524.

55 [0044] pKCT4 produced as described above had a structure where the replication origin derived from pHM1519

## EP 1 038 970 A2

modified to be temperature sensitive was inserted into *KpnI* site of pHSG399. A fragment containing a temperature sensitive replication origin was obtained by digesting pKCT4 with *KpnI*, blunt-ended by using Blunting Kit (produced by Takara Shuzo), and ligated to pHSG $\Delta$ gluAD digested with *HincII* to obtain pT $\Delta$ AD (Fig. 1).

5 (2) Construction of plasmid for disruption of *gluA* gene

[0045] In order to create a coryneform bacterium in which only the *gluA* gene was disrupted, a plasmid for disruption of the *gluA* gene was constructed.

10 [0046] A DNA fragment of about 1500 bp containing the *gluA* gene was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by PCR utilizing oligonucleotides having the nucleotide sequences shown as SEQ ID NOS: 3 and 5 as primers, and the amplified fragment was digested with *EcoRI*, and ligated to pHSG299 (produced by Takara Shuzo) digested with *EcoRI* by using T4 ligase (produced by Takara Shuzo) to obtain a plasmid pHSGgluA.

15 [0047] Then, the 5' region and the 3' region of the *gluA* gene and the vector segment, except for the internal region of the *gluA* gene, were amplified by PCR utilizing oligonucleotides having the nucleotide sequences shown as SEQ ID NOS: 6 and 7 as primers, and pHSGgluA as a template. The aforementioned primers were designed so that it should contain a *BglII* recognition sequence. The amplified fragment was digested with *BglII*, and allowed to cause self-ligation in the presence of T4 ligase to obtain a plasmid pHSG $\Delta$ gluA. This plasmid contained deletion of about 250 bp of internal sequence among the about 730 bp open reading frame of *gluA*, and had a structure where the 5' region and the 3' region were ligated in-frame.

20 [0048] Then, in order to make pHSG $\Delta$ gluA autonomously replicable in coryneform bacteria, a temperature sensitive replication origin derived from a plasmid autonomously replicable in coryneform bacteria was introduced into the unique *KpnI* cleavage site in pHSG $\Delta$ gluA. Specifically, pKCT4 was digested with *KpnI* to obtain a DNA fragment containing a replication origin, and the obtained fragment was inserted into *KpnI* site of pHSG $\Delta$ gluA to obtain pT $\Delta$ A (Fig. 2).

25 (3) Creation of *gluABCD* gene-disrupted strain and *gluA* gene-disrupted strain

[0049] The plasmids for disrupting genes obtained as described above, pT $\Delta$ AD and pT $\Delta$ A, were introduced into a wild-type strain, *Brevibacterium lactofermentum* ATCC13869 strain, by using the electric pulse method to obtain ATCC13869/pT $\Delta$ AD and ATCC13869/pT $\Delta$ A. Gene disruption was performed by using these transformant strains.

30 [0050] Specifically, ATCC13869/pT $\Delta$ AD and ATCC13869/pT $\Delta$ A were cultured at 25°C in CM2B broth for 24 hours with shaking, and inoculated to CM2B medium containing 25  $\mu$ g/ml of kanamycin. Strains into which the plasmids were introduced were obtained as strains that formed colonies at 34°C, at which temperature the temperature sensitive replication origin did not function. Then, the strains that became sensitive to kanamycin at 34°C were obtained by the replica method. Chromosome DNA of these sensitive strains was obtained in a conventional manner. The structures of the *gluABCD* gene and the *gluA* gene on the chromosome was examined by PCR and sequencing to confirm that these genes should be replaced with those of the deletion type, and the strains containing the deletion type genes were designated as  $\Delta$ AD strain and  $\Delta$ A strain, respectively.

35 [0051] The  $\Delta$ AD strain and the  $\Delta$ A strain were given with private numbers of AJ13587 and AJ13588, respectively, and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (postal code: 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on March 23, 1999, and received accession numbers of FERM P-17327 and FERM P-17328, respectively, and then, transferred from the original deposit to international deposit based on Budapest Treaty on February 14, 2000, and have been deposited as deposit numbers of FERM BP-7028 and FERM BP-7029, respectively.

45 (4) Evaluation of L-glutamic acid-producing ability of strains  $\Delta$ AD and  $\Delta$ A

40 [0052] Culture of the strains ATCC13869,  $\Delta$ AD and  $\Delta$ A for the production of L-glutamic acid was performed as follows. These strains that had been refreshed by culture in a CM2B plate medium were cultured in two kinds of mediums, a medium containing 80 g of glucose, 1 g of  $\text{KH}_2\text{PO}_4$ , 0.4 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.01 g of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g of  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 ml of soybean hydrolysate solution, 200  $\mu$ g of thiamin hydrochloride, 3  $\mu$ g of biotin, and 50 g of  $\text{CaCO}_3$  in 1 L of deionized water (prepared at pH 8.0 by using KOH), and a medium further containing 50 g/L of L-glutamic acid in the foregoing medium, at 31.5°C. After the cultivation, the amounts of accumulated L-glutamic acid in the mediums, and absorbance at 620 nm of the mediums diluted 51 times were measured. The results obtained for the medium with no addition of L-glutamic acid were shown in Table 1. The results obtained for the medium added with L-glutamic acid were shown in Table 2.

## EP 1 038 970 A2

Table 1

Strain	OD <sub>620</sub>	L-glutamic acid (g/L)	Yield (%)
ATCC 13869	0.937	40.8	50.4
$\Delta$ AD	1.127	36.3	44.9
$\Delta$ A	0.766	44.3	54.8

Table 2

Strain	OD <sub>620</sub>	L- glutamic acid (g/L)*	Yield (%)
ATCC 13869	0.845	29.5	43.9
$\Delta$ AD	0.927	30.0	44.6
$\Delta$ A	0.749	32.0	47.6

\* The amounts do not include the L-glutamic acid added to the medium.

[0053] These results show that the accumulation amount and yield of L-glutamic acid were improved for both of the  $\Delta$ A strain and the  $\Delta$ AD strain when the medium contained L-glutamic acid at a high concentration. Further, the yield of L-glutamic acid was improved by the  $\Delta$ A strain even in the medium not containing L-glutamic acid.



EP 1 038 970 A2

## SEQUENCE LISTING

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EP 1 038 970 A2

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EP 1 038 970 A2

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### Claims

1. A method for producing L-glutamic acid, comprising the steps of culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.
2. The method according to claim 1, wherein the L-glutamic acid uptake system is encoded by the *gluABCD* operon.
3. The method according to claim 2, wherein at least one of expression products of the *gluABCD* operon is deleted in the coryneform bacterium.
4. The method according to claim 3, wherein at least *gluA* is deleted in the coryneform bacterium.
5. The method according to claim 4, wherein all of *gluA*, *gluB*, *gluC* and *gluD* are deleted in the coryneform bacterium.

EP 1 038 970 A2

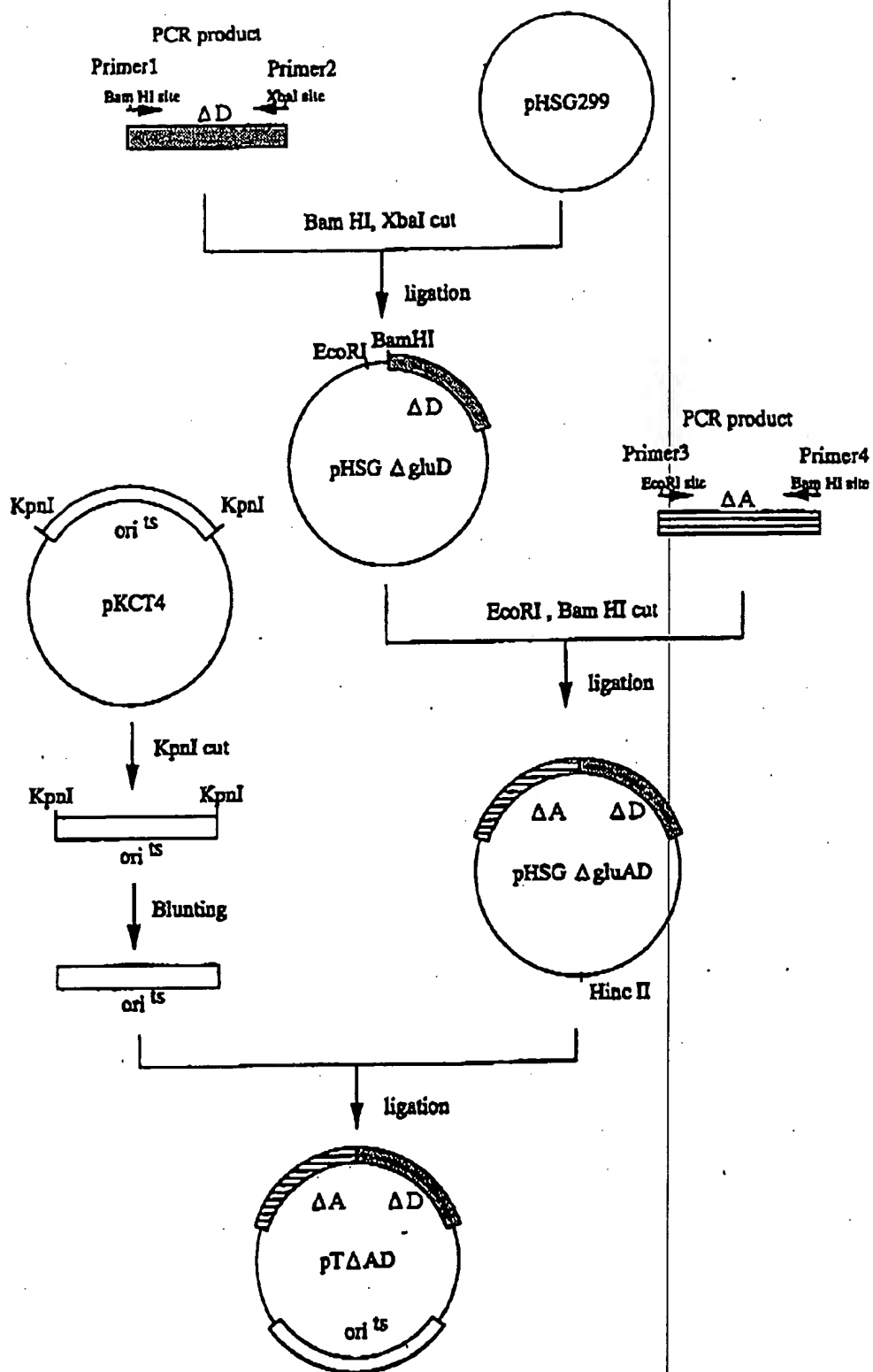


Fig. 1

EP 1 038 970 A2

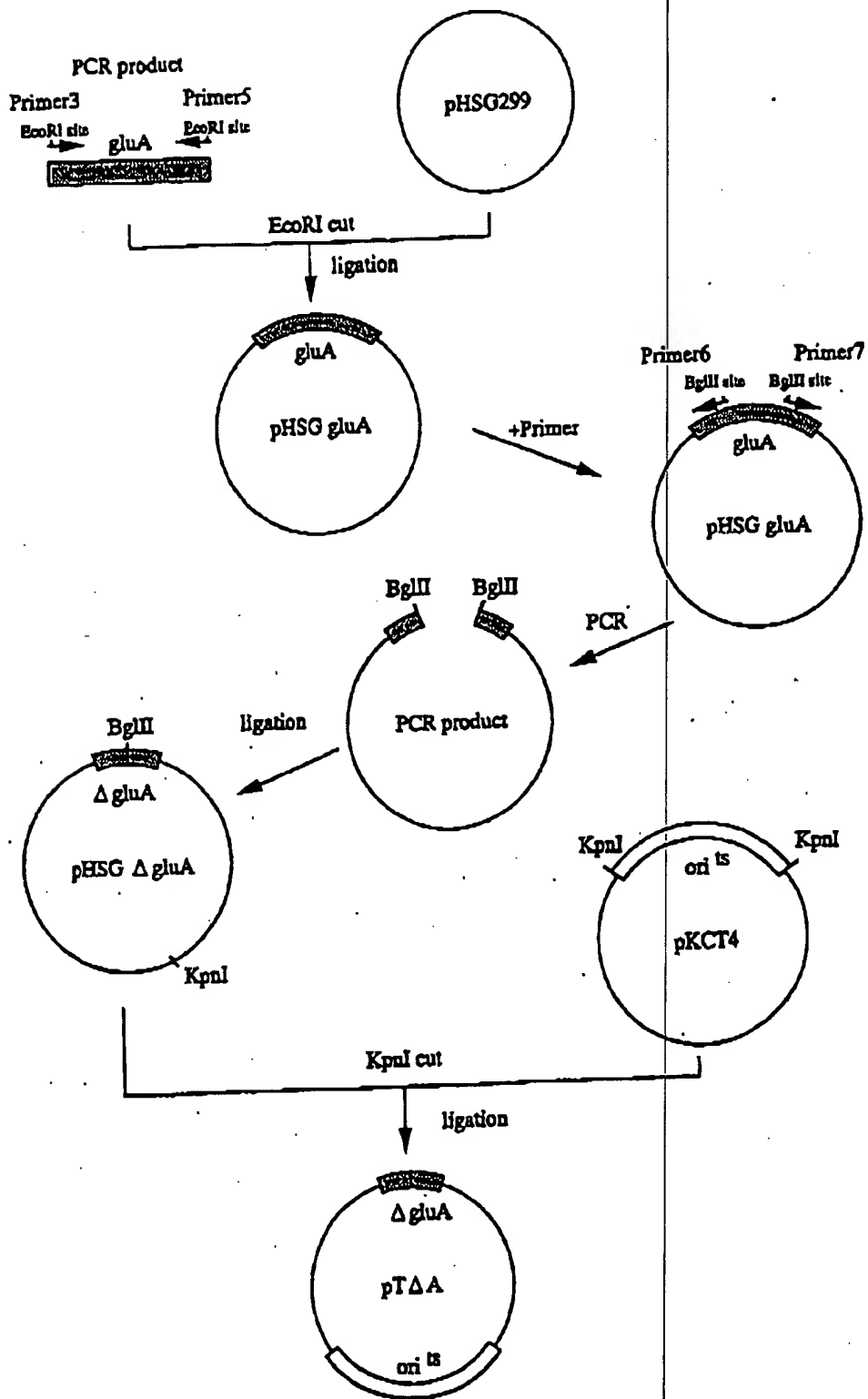


Fig. 2

<b>(12) PATENT</b>		<b>(11) Application No. AU 199719218 B2</b>	
<b>(19) AUSTRALIAN PATENT OFFICE</b>		<b>(10) Patent No. 724536</b>	
<b>(54) Title</b> Process for the microbial production of amino acids by boosted activity of export carriers			
<b>(51)<sup>5</sup> International Patent Classification(s)</b> C12N 015/00			
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19540222	1995.12.22	DE	
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<b>(71) Applicant(s)</b> Forschungszentrum Jülich GmbH			
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 AOJP DATE 11/09/97 PCT NUMBER PCT/DE96/02485



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(21) Internationales Aktenzeichen: PCT/DE96/02485 (22) Internationales Anmeldedatum: 18. Dezember 1996 (18.12.96) (30) Prioritätsdaten: 195 48 222.0 22. December 1995 (22.12.95) DE (71) Anmelder (für alle Bestimmungsstaaten ausser US): FORSCHUNGSZENTRUM JÜLICH GMBH [DE/DE]; Wilhelm-Johnen Strasse, D-52425 Jülich (DE). (72) Erfinder; and (75) Erfinder/A anmelder (nur für US): VRLIC, Marina [DE/DE]; Steinstrasser Allee 60, D-52428 Jülich (DE). EGGELING, Lothar [DE/DE]; Eisenkamp 6, D-52428 Jülich (DE). SAHM, Hermann [DE/DE]; Wendellaustrasse 71, D-52428 Jülich (DE). (74) Gemeinssamer Vertreter: FORSCHUNGSZENTRUM JÜLICH GMBH; Rechts- und Patentabteilung, D-52425 Jülich (DE).		(81) Bestimmungsstaaten: AU, BR, CA, CN, JP, KR, MX, RU, US, europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Veröffentlicht Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.	
(54) Title: PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS BY BOOSTED ACTIVITY OF EXPORT CARRIERS (54) Beschreibung: VERFAHREN ZUR MIKROBIELLEN HERSTELLUNG VON AMINOSÄUREN DURCH GESTEIGERTE AKTIVITÄT VON EXPORTCARRIERN			
		LYSINE SECRETION Lysinsekretion pMV6-3 - pMV6-5-26 + pMV7-2 - pMV3-7 - pMV2-3 +	
(57) Abstract  The invention pertains to a process for the microbial production of amino acids. The process in question involves boosting the export carrier activity and/or export gene expression of a micro-organism which produces the desired amino acid. According to the invention, it was found that a single specific gene is responsible for the export of a given amino acid, and on that basis a process for the microbial production of amino acids, involving the controlled boosting of the export gene expression and/or export carrier activity of a micro-organism which produces the amino acid in question, has been developed for the first time. The boosted expression or activity of the export carrier resulting from this process increases the secretion rate and thus increases transport of the desired amino acid.			

FJ 122

PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS  
BY BOOSTED ACTIVITY OF EXPORT CARRIERS

The invention relates to a process for the microbial production of amino acids according to claims 1 to 20, export genes according to claims 21 to 26, regulator genes according to claims 29 and 30, vectors according to claims 31 to 33, transformed cells according to claims 34 to 40, membrane proteins according to claims 41 and 42 and uses according to claim 43 and 48.

Amino acids are of high economical interest and there are many applications for the amino acids: for example, L-lysine as well as L-threonine and L-tryptophan are needed as feed additives, L-glutamate as seasoning additive, L-isoleucine, and L-tryosine in the pharmaceutical industry, L-arginine and L-isoleucine as medicine or L-glutamate and L-phenylalanine as a starting substance for the synthesis of fine chemicals.

A preferred method for the manufacture of these different amino acids is the biotechnological manufacture by means of microorganisms; since, in this way, the biologically effective and optically active form of the respective amino acid is directly obtained and simple and inexpensive raw materials can be used. As microorganisms, for example, *Carynebacterium glutamicum* and its relatives ssp. *flavum* and ssp. *lactofermentum* (Liebl et al; Int. J-System Bacteriol (1991) 41:255-260) as well as *Escherichia coli* and related bacteria can be used.

However, these bacteria produce the amino acids only in the amounts needed for their growth such that no excess amino acids are generated and are available. The reason for this is that in the cell the biosynthesis of the amino acids is con-





trolled in various ways. As a result, different methods of increasing the formation of products by overcoming the control mechanisms are already known. In these processes, for example, amino acid analogs are utilized to render the control of the biosynthesis ineffective. A method is described, for example, wherein *Coarnebacterium* strains are used which are resistant to L-tyrosine and L-phenylalanine analogs (JP 19037/1976 and 39517/1978). Also methods have been described in which bacteria resistant to L-lysine and also to L-threonine analogs are used in order to overcome the control mechanisms (EP 0 205 849 B1, UK patent application GB 2 152 509 A).

Furthermore, microorganisms constructed by recombinant DNA techniques are known wherein the control of the biosynthesis has also been eliminated by cloning and expressing the genes which code for the key enzymes which cannot be feed-back inhibited any more. For example, a recombinant L-lysine producing bacterium with plasmid-coded feedback-resistant aspartate kinase is known (EP 0381527). Also, a recombinant L-phenylalanine producing bacterium with feedback resistant prephenate dehydrogenase has been described (JP 124375/1986; EP 0 488 424). In addition, increased amino acid yields have been obtained by overexpression of genes which do not code for feed-back-sensitive enzymes of the amino acids synthesis. For example, the lysine formation is improved by increased synthesis of the dihydrodipicolinate synthase (EP 0 197 335). Also, the threonine formation is improved by increased synthesis of threonine dehydratase (EP 0 436 886 A1).

Further experiments for increasing the amino acid production aim at an improved generation of the cellular primary metabolites of the central metabolism. In this connection, it is known that the overexpression of the transketolase achieved by recombinant techniques improve the product generation of L-tryptophan, L-tyrosine or L-phenylalanine (EP 0 600 463 A2).



Furthermore, the reduction of the phosphoenol pyruvate carboxylase activity in *Corynebacterium* provides for an improvement in the generation of aromatic amino acids (EP 0 331 145).

All these attempts to increase the productivity have the aim to overcome the limitation of the cytosolic synthesis of the amino acids. However, as a further limitation basically also the export of the amino acids formed in the interior of a cell into the culture medium should be taken into consideration. As a result, it has been tried to improve this export and, consequently, the efficiency of the amino acid production. For example, the cell permeability of the *Corynebacterium* has been increased by biotin deficiency, detergence or penicillin treatment. However, these treatments were effective exclusively in the production of glutamate, whereas the synthesis of other amino acids could not be improved in this manner. Also, bacteria strains have been developed in which the activity of the secretion system is increased by chemical or physical mutations. In this way, for example, a *Corynebacterium glutamicum* strain has been obtained which has an improved secretion activity and is therefore especially suitable for the L-Lysine production. (DE 02 03 320).

Altogether, the attempts to increase the secretion of amino-acids formed within the cell have all in common that an increase efflux of amino acids on the basis of the selected non-directed and non-specific methods could be achieved only accidentally.

Solely in the German patent application No. 195 23 279.8-41, a process is described which provides for a well-defined increase of the secretion of amino acids formed internally in a cell by increasing the expression of genes coding for the import of amino acids. The understanding on which this process was based, that is, the cell utilizes import proteins for the export of amino acids as well as the fact that by nature micro-



organisms do not generate and release excess amino acids lets one assume that export genes or proteins specific for the amino acid transport do not exist, but that the amino acids are excreted by way of other export systems.

The export systems known so far export poisonous metal ions, toxic antibiotics and higher molecular toxins. These export systems are relatively complex in their structure. Generally, membrane proteins of the cytoplasmic membrane are involved which however cause only a partial reaction of the export so that presumably additional extra cytoplasmic support proteins are needed for the transport (Dink, T. et al., A family of large molecules across the outer membranes of gram-negative bacteria., J. Bacteriol. 1994, 176: 3825-3831). Furthermore, it is known that, with the sec-dependent export system for extra-cellular proteins, at least six different protein components are essential for the export. This state-of-the-art suggests that also the systems, which are responsible for the export of amino acids, but which are not known so far comprise several protein components or respectively, several genes are responsible for the export of amino acids. A hint in this direction could be the various mutants which are defective in the lysine export as described by Vrylic et al., (J. Bacteriol (1995) 177:4021-4027).

It has now been found surprisingly that only a single specific gene is responsible for the export of amino acids so that, in accordance with the invention, for the first time a method for the microbial manufacture of amino acids is provided wherein clearly the export gene expression and/or the export carrier activity of a microorganism producing amino acids is increased. The increased export expression or respectively, activity of the export carrier resulting from this process leads to an increased secretion rate so that the export of the respective amino acid is increased. The microorganisms so



modified also accumulate an increased part of the respective amino acid in the culture medium.

For an increase in the export carrier activity especially the endogenic activity of an amino acid producing microorganism is increased. An increase of the enzyme activity can be obtained for example by an increased substrate consumption achieved by changing the catalytic center or by eliminating the effects of enzyme inhibitors. An increased enzyme activity can also be caused by an increased enzyme synthesis for example by gene amplification or by eliminating factors which inhibit the enzyme biosynthesis. The endogene export activity is increased preferably by mutation of the endogenic export gene. Such mutations can be generated either in an uncontrolled manner in accordance with classic methods as for example by UV irradiation or by mutation causing chemicals or in a controlled manner by gene-technological methods such as deletion(s) insertion(s) and/or nucleotide exchange(s).

The export gene expression is increased by increasing the number of gene copies and/or by increasing regulatory factors which positively affect the export gene expression. For example, a strengthening of regulatory elements takes place preferably on the transcription level by increasing particularly the transcription signals. This can be accomplished for example in that, by changing the promoter sequence arranged before the structure gene, the effectiveness of the promoter is increased or by completely replacing the promoter by more effective promoters. An amplification of the transcription can also be achieved by accordingly influencing a regulator gene assigned to the export gene as will be explained further below. On the other hand, an amplification of the translation is also possible, for example, by improving the stability of the m-RNA.

To increase the number of gene copies the export gene is installed in a gene construct or, respectively, in a vector,



preferably, a vector with a small number of copies. The gene construct includes regulatory gene sequences, which are specifically assigned to the export gene, preferably such sequences which reinforce the gene expression. The regulatory gene sequences comprise a nucleotide sequence which codes for the amino acid sequence given in table 1 or the allele variations thereof or respectively, a nucleotide sequence 954 to 82 according to table 2 or a DNA sequence which is effective essentially in the same manner.

Allele variations or, respectively, equally effective DNA sequences comprise particularly functional derivatives which can be obtained by deletion(s) insertion(s) and/or substitution(s) of nucleotides of corresponding sequences, wherein however the regulator protein activity or function is retained or even increased. In this way, the effectiveness of the interaction of the regulatory protein to the DNA of the export gene to be regulated can be influenced by mutating the regulatory gene sequence such that the transcription is strengthened and, consequently, the gene expression is increased. In addition, also so-called enhancers may be assigned to the export gene as regulatory sequences whereby, via an improved correlation between RNA polymerase and DNA, also the export gene expression is increased.

For the insertion of the export gene into a gene construct, the gene is preferably isolated from a microorganism strain of the type *Corynebacterium* and, with the gene construct including the export gene, a microorganism strain, especially *Corynebacterium*, producing the respective amino acid is transformed. The isolation and transformation of the respective transport gene occurs according to the usual methods. If a transport gene is isolated and cloned from *Corynebacterium* then for example, the method of homologous complementation of an export defective mutant is suitable (J.Bacteriol. (1995)177:



4021-4027). If a direct cloning of the structure gene is not possible vector sequences may first be inserted into the transport gene whereupon it is isolated by way of "plasmid rescue" in the form of inactive fragments. For the process according to the invention genes from the *C. glutamicum* ATCC 13032 or *C. glutamicum* ssp. *flavum* 14067 or also, *C. glutamicum* ssp. *lacto fermentum* ATCC 13869 are particularly suitable. The isolation of the genes and their in-vitro recombination with known vectors (Appl. Env. Microbial (1989)55: 684-688; Gene 102(1991)93-98) is followed by the transformation into the amino acid producing strains by electroporation (Liebl et al. (1989)FEMS Microbiol Lett. 65: 299-304) or, conjugation (Schäfer et al. (1990) J. Bacteriol. 172:1663-1666). For the transfer, preferably vectors with low numbers of copies are used. As host cells, preferably such amino acid producers are used which are deregulated in the synthesis of the respective amino acids and/or which have an increased availability of central metabolism metabolites.

After isolation, export genes with nucleotide sequences can be obtained which code for the amino acid sequences given in table 3 or for their allele variations or, respectively, which include the nucleotide sequence of 1016 to 1725 according to table 2 or a DNA sequence which is effective essentially in the same way. Also here, allele variations or equally effective DNA sequences include particularly functional derivatives in the sense indicated above for the regulatory sequences. These export genes are preferably used in the process according to the invention.

One or several DNA sequences can be connected to the export gene with or without attached promoter or respectively, with or without associated regulator gene, so that the gene is included in a gene structure.

By cloning of export genes, plasmids or, respectively,



vectors can be obtained which contain the export gene and which, as already mentioned, are suitable for the transformation of an amino acid producer. The cells obtained by transformation which are mainly transformed cells from *Corynebacterium*, contain the gene in reproducible form, that is, with additional copies on the chromosome wherein the gene copies are integrated at any point of the genome by homologous recombination and/or on a plasmid or respectively, vector.

A multitude of sequences is known which code for membrane proteins of unknown function. By providing in accordance with the invention export genes such as the export gene with the nucleotide sequence of nucleotide 10165 to 1725 in accordance with table 2 or respectively, the corresponding export proteins for example that with the amino acid sequence according to table 1, it is now possible to identify by sequence comparison membrane proteins, whose function is the transport of amino acids. The export gene identified in this way can subsequently be used to improve the amino acid production in accordance with the process of the invention.

The membrane proteins known from the state-of-the-art generally include 12, some also only 4 transmembrane helices. However, it has now been found surprisingly that the membrane proteins responsible or suitable for the export of amino acids include 6 transmembrane helices (see for example, the amino acid sequence of an export protein listed in the table 3, wherein the 6 transmembrane areas have been highlighted by underlining). Consequently, there is a new class of membrane proteins present which has not yet been described.

Examples:

a) Cloning of an export gene and cloning of a regulator of *Corynebacterium glutamicum*.

Chromosomal DNA from *C. glutamicum* R127 (FEMS Microbiol Lett. (1989) 65:299-304) was isolated as described by Scharzer



et al. (Bio/Technology (1990) 9:84-87). The DNA was then split with the restriction enzyme Sau3A and separated by saccharose gradient centrifugation as described in Sambrook et al. (Molecular cloning, A laboratory manual (1989) Cold Spring Harbour Laboratory Press). The various fractions were analyzed gel electrophoretically with respect to their size and the fraction with a fragment size of about 6 - 10kb was used for the ligation with the vector pJCl. In addition, the vector pJCl was linearized with BamHI and dephosphorylized. Five ng thereof was ligated with 20ng of the chromosomal 6-10 kb fragments. With the whole ligation preparation, the export defective mutant NA8 (J. Bacteriol. (1995)177:4021-4027) was transformed by electroporation (FEMS Microbiol Lett(1989)65:299 - 304). The transformants were selected for LBHIS(FEMS Microbiol. Lett. (1989)65:299-304) with 15µg kanamycin per ml. These transformants were subjected to extensive plasmid analyses in that 200 of the altogether 4500 clones obtained were individually cultivated and their plasmid content and size was determined. On average, about half of the kanamycin-resistant clones carried a recombinant plasmid with an insert of the average size of 8kb. This provides for a probability of 0.96 for the presence of any gene of *C. glutamicum* in the established gene bank. The 4500 obtained transformants were all individually checked for renewed presence of lysine secretion. For this purpose, the system described by Vrljic for the induction of the L-lysine excretion in *Corynebacterium glutamicum* was utilized (J. Bacteriol (1995) 177:4021-4027). For this purpose, so-called minimal-medium-indicator plates were prepared, which contained per liter 20g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5g uric acid, 1g KH<sub>2</sub>PO<sub>4</sub>, 1 g K<sub>2</sub>HPO<sub>4</sub>, 0.25g MgSO<sub>4</sub>·x7H<sub>2</sub>O, 42 g morpholino propane sulfonic acid, 1ml CaCl<sub>2</sub> (1g/100ml), 750 ml dest., 1 ml Cg trace salts, 1 ml biotin (20µg/100l), pH7, 4% glucose, 1.8mg protocatechuic acid, 1 mg FeSO<sub>4</sub> · x 7 H<sub>2</sub>O, 1 mg MnSO<sub>4</sub> · x H<sub>2</sub>O, 0.1 mg ZnSO<sub>4</sub> · x 7H<sub>2</sub>O,





0.02mg CuSO<sub>4</sub>, 0.002mg NiCl<sub>2</sub> x 6H<sub>2</sub>O, 20 g agar-agar, as well as 10<sup>7</sup> cells/ml of the lysine-auxotrophene *C. glutamicum* mutant 49/3. The original 4500 transformants were all individually pinned, by toothpicks onto the indicator plates with, in each case, a check of the original non-excretor NA8 (J.Bacteriol (1995)177:4021-4027) and the original strain R127. At the same time, always 2 plates were inoculated of which only one contained additionally 5mM L-methionine in order to induce the lysine excretion in this way. The indicator plates were incubated at 30°C and examined after 15, 24 and 48 hours. In this way, altogether 29 clones were obtained which showed on the indicator plate provided with methionine a growth court by the indicator strain 49/3. The clones were examined individually and then again as described above, for reestablishment of the growth court. In this way, the two clones NA8 pMV8-5-24 and NA8 pMV6-3 were obtained which had again received the capability to excrete lysine.

From these clones, plasmid preparations were performed as described in Schwarzer et al. (Bio/Technology (1990)9; 84-87). By retransformation in NA8, the plasmid-connected effect of the excretion of L-lysine was confirmed. Both plasmids were subjected to a restriction analysis. Plasmid pMV8-5-24 carries an insert of 8.3 kb, and pMV6-3 one of 9.5 kb. The physical character of the inserts is shown in Fig. 1.

b) Subcloning of an DNA fragment which reconstitutes the lysine export.

From the insert of the plasmid pMV6-3 individual subclones were prepared utilizing the restriction severing point as determined. In this way, the 3.7 kb XhoI-SalI-fragment, the 2.3 kb BamHI-fragment and the 7.2 kb BamHI fragment were ligated with the correspondingly severed and treated vector pUC1 (Mol Gen. Genet. (1990)220: 478-480). With the ligation products *C. glutamicum* NA8 was directly transformed, the transformants were



tested for having the lysine excretion properties and the presence of the subclone was confirmed by plasmid preparation and restriction analysis. In this way, the strain with plasmid pMV2-3 (Fig. 1) was obtained as smallest subclone. This fragment resulting in lysine export contains as insert the 2.3kb Bam fragment from pMV6-3.

c) Sequence of the lysine export gene *lys E* and its regulators *lysG*.

The nucleotide sequence of the 2.3kb BamHI fragment was performed according to the dideoxy-chain termination method of Sanger et al. (Proc. Natl. Acad. Sci USA(1977) 74:5463-5467) and the sequencing reaction with the Auto Read Sequencing kit from Pharmacia (Uppsala, Sweden). The electrophoretic analysis occurs with the automatic laser-fluorescence DNA sequencing apparatus (A.L.F) from Pharmacia-LKB(Piscataway, NJ, USA). The nucleotide sequence obtained was analyzed by the program packet HUSAR (Release 3.0) of the German Cancer Research Center (Heidelberg). The nucleotide sequence and the result of the analysis is presented in Fig. 2. The analysis results in two fully open reading frames (ORF) on the sequenced DNA piece. ORF1 codes for a protein with a length of 236 amino acids, ORF2 codes for a protein with a length of 290 amino acids. The protein derived from ORF1 includes an accumulation of hydrophobic amino acids as they are characteristic for membrane-embedded proteins. The detailed analysis of the distribution of the hydrophobic and hydrophilic amino acids by the programs PHD.HTM (Protein Science(1995)4:521-533) is shown in table 3. It is apparent therefrom that the protein contains six hydrophobic helix areas which extend through the membrane. Consequently, this protein is the searched for exporter of the amino acid L-lysine. The corresponding gene will therefore be designated below as *lysE*. In table 2, it is marked accordingly. ORF2 is transcribed in a direction opposite to ORF1. The sequence



analysis shows that ORF2 has a high identity with regulator genes which are combined as a single family (Ann Rev Microbiol(1993) 597-626). Genes of this family regulate the expression processes of the various genes involved in catabolic or anabolic processes in a positive way. For this reason, ORF2 will below be designated as lysG (Govern=regulating). Because of the coordination and because lysE could be cloned (see a)) and subcloned (see b)) together with lysG, lysG is regulator of lysE and consequently also participates in the lysine export. The gene lysG and the amino acid sequence derived therefrom are also shown in table 2 and, respectively, table 1.

d) Identification of an unknown membrane protein from *Escherichia coli* by sequence comparison.

With the established sequences according to table 3 already existing sequence banks can be searched in order to assign the proteins derived in this way from sequenced areas a certain function. Correspondingly, the amino acid sequence of the lysine exporters consisting of *C. glutamicum* were compared with derivated protein sequences of all the DNA sequences deposited there utilizing the program packet HUSAR (Release 3.0) of the German Cancer Research Center (Heidelberg). A high homology of 39.3% identical amino acids and 64.9% similar amino acids was found to a single sequence of so far unknown function of *E. coli*.

The comparison is shown in Fig. 2. The open read frame of *E. coli* so far not characterized is consequently identified by way of this process as an amino export gene.

e) Increased export of intracellularly accumulated L-lysine.

The strain *C. glutamicum* NA8 (J. Bacteriol(1995) 177: 4021-4027) was transformed with plasmid pMV2-3 and the L-lysine excretion of the strains was compared. For this purpose, NA8 and NA8pMV2-3 in complex medium were utilized as described in



Vrljic et al. (J. Bacteriol (1995) 177:4021-40277) and the fermentation medium CGXII (Bacteriol (1993) 175:5595-5603) were each separately inoculated. The medium additionally contained 5mM L-methionin in order to induce the intracellular L-lysine biosynthesis. After cultivation for 24 hours at 30°C on a rotary vibrator at 140 rpm, the cell internal and external L-lysine determinations were performed. For the cell-internal determination silicon oil centrifugations were performed (Methods Enzymology LV(1979) 547-567); the determination of the amino acids occurred by high pressure liquid chromatography (J. Chromat (1983) 266:471-482). These determinations were performed at different times as indicated in Fig. 3. In accordance with the process used the retained cell internal L-lysine is excreted also by pMV2-3 to a greater degree and is accumulated. Accordingly, also the cell internally present L-lysine is greatly reduced. Consequently, the utilization of the newly discovered and described exporter represents a process for greatly improving the L-lysine production.

f) Increased accumulation of L-lysine by lysE or LysEG.

From the subclone pMV2-3 which contains the sequenced 2374bp Bam HI-fragment in pJCI (see figure 1), the lysE carrying 1173 bpPvuII fragment was ligated in pZ1 (Appl. Env. Microbiol(1989)55:684-688) according to the sequence information and in this way, the plasmid plysE was obtained. This plasmid as well as the lysE lysG carrying plasmid pMV2-3 was introduced into C. glutamicum strain d by electroporation wherein the chromosomal areas were deleted. The obtained strains C. glutamicum d pMV2-3, C. glutamicum d plysE, C. glutamicum pJCI were, as described under e) precultivated on a complex medium, then cultivated in production minimal medium CGx11 together with 4% glucose and 5mM L-methionin and samples were taken to determine the accumulated lysine. As apparent from Fig. 4 with lysE lysG an increase of the lysine accumulation with respect



to a control sample is achieved. With plysE an extraordinarily increased accumulation of from 4.8 to 13.2 mM L-lysine is achieved with this method.

#### LEGENDS OF THE TABLES AND FIGURES

Table 1: The amino acid sequence of the lysine exporter regulator from *Corynebacterium glutamicum* with the helix-turn-helix motive typical for DNA-binding proteins.

Table 2(three pages): The nucleotide sequence of *C. glutamicum* coding for the lysine exporter and lysine export regulators.

Table 3: the amino acid sequence of the lysine exporter from *Corynebacterium glutamicum* with the identified transmembrane helices TMH1 to TMH6.

Figure 1: the fragments in pMV6-3 and pMV8-5-24 obtained by the cloning which cause the lysine secretion and the subclone pMV2-3 made from pMV6-3, which also causes the lysine secretion and which was sequenced. B, BamHI; Sm, SmaI; Se, SacI; Sl, Sal I, II, HindII; X, XhoI.

Figure 2: Comparison of the derivated amino acid sequence of LysE from *C. glutamicum* (above), with a gene product of so far unknown function from *Escherichia coli* (below), which is identified thereby as export carrier.

Fig. 3: Increased lysine export by pMV2-3 with *C. glutamicum* NA8. On top, the control with low excretion and cell-internal backup of lysine up to about 150mM. Below, the high secretion caused by pMV2-3 with cell internally only small backup of about 30mM.

Figure 4: the increase of the lysine accumulation in *C. glutamicum* by lys E lys G(pMV2-3) (middle curve); and the accumulation caused by lysE(plysE) (upper curve).



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**EDITORIAL NOTE**

**No: 19218/97**

**The following five pages are unnumbered**

**(Table 1 to Table 3)**

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1 MNPIQLDTLL SIIDEGSEEG ASLALSISPS AVSQRVKALE HHVGRVLVSR

Helix-Turn-Helix-Motiv

51 TOPAKATEAG EVLVQAARKM VLIQAETKAQ LSGRLAEIPL TIAINADSLS

101 TWFPFVENEV ASWGGATLTL RLEDEAHTLS LLRRGDVLGA VTREANPVAG

151 CEVVELGTMR HLAIATPSLR DAYMVDGKLD WAAMPVLREG PKDVLQDRDL

201 DGRVDGPVGR RRVSIVPSAE GFGEAIRRGL GWGLLPETQA APLKAGEVI

251 LLDEIPIDTP MYWQRWRLES RSLARLTDV VDAAIEGLRP

Table 1





1 MNPIQLDTLL SIIDEGSFEG ASLALSISPS AVSQRVKALE HHVGRVLVSR

Helix-Turn-Helix-Motiv

51 TOPAKATEAG EVLVQAARKM VLLQAEITKAQ LSGRLAEIPL TIAINADSL

101 TNEPPVENEV ASWGGATLTL RLEDEAHTLS LLRRGDVLGA VTREANPVAG

151 CEVVELGTMR HLAIATPSLR DAYMVDGKLD WAAMPVLREG PKDVLQDRDL

201 DGRVDGPVGR RRVSIVPSAE GFGEAIRRGL GWGLLPETOA APMLKAGEVI

251 LLDEIPIDTP MYQWRWLES RSLARLTDV VDAIEGLRP

Table 1



BCGS 960  
<---LysG  
CTTGGACGGAGTACTTAACTCTCGTTTACAGGTCACTTACCCCAAGA-----5'  
5'---TCCCTTCATCAATGATTGAGGCAAGGTGTCCASTTGAATCGGTTTCAAGAAGCT  
F S G E D I I S L L T D L Q I P N M  
HB9 1020  
ATATTAAACCATGTGTAAGAACCAATCATTTTACTTAAGTACTTCCATAGGTACCAAGCT  
M V  
LysR-->  
GATCATGGAAATCTTCATTACGGCTCTGCTTTTGGGGGGAGTCTTTTACTGTCCATGGG 1080  
I M E I F I T G L L L G A S L L L S I G  
ACCGCAAAATGTACTGGTGAATTAACCAAGGAATTAAGCCGGAAGGACTCATTCGGGTTX 1140  
P Q N V L V I K Q G I K R E G L I A V L  
TCTCGTGTGTTTAATTCTGAGCTCTTTTGTTCATCGCCCGCCACCTTGGCGTTGATCT 1200  
L V C L I S D V F L P I A G T L G V D L  
TTTGTCCAAATGCGCCGCGCATCTGCTCGATATTATGCGCTGGGGTGGCATCGCTTACTT 1260  
L S N A A P I V L D I H R W G G I A Y L  
GTIATGCTTTGCCGTGATGGCAGCAAGGACGCGCATGACAAACAGGTGCAAGCGCCACA 1320  
I W P A V M A A K D A H T N K V K A P Q  
GATCATTGAACAAACAGAACCAACCGTCCCGATGACAGCTTTTGGCGGTTTGGCGGT 1380  
I I E E T E P T V P D D T P L G G S A V  
>>>>>> > < <<<<<<<  
GGCCACTGACACCGCGCAACCGGTGGGGGTGGAGGTGAGGCTCGATAGCAGCGGTTT 1440  
A T D T R N R V R V E V S V D K Q R V W  
GGTAAAGCCCATGTTGATGGCAATCTGCTGACCTCGTTGAACCGGAATGCTATTGG 1500  
V K P M L M A I V L T W L N P N A Y L D  
CGCGTTTGTGTTTATCGCGCGGCTCGCGCCGCAATACGGCAGACCCGACCGTGGATTT 1560  
A F V P I G G V G A Q Y G D T G R W I P  
CGCGCTTGGCGGTTTGGCGGCAAGGCTGATCTGTTTCCGCTCGTGGGTTTGGCGGAGC 1620  
A A G A P A A S L I W P P L V G F G A A  
AGCATTTGACGCGCGCTGTCCAGCCCAAGGTCTGCGCTCGATCAAGTCTGCTGCTGCG 1680  
A L S R P L S S P K V W R W I N V V V A



1740  
5' CTACTGGCGTAACGGGTACTTTGACTAGAACTACCCAAATCAAAAGCCGCCAAA  
AGTTGTGATGACCGCATTTGGCCATCAAACTGATGTTGATGGTTTGGGGG 5'  
V V M T A L A I K L M L H G -  
1780  
CCTTACCCACCGGACCGGGTTTACAACTACGGCCGAGACCCCTTTAGAGTAGTAGGG  
S D I A K A W I N I G A D H S I E D I A  
1800  
CAGCTTGAGCGCGAGTCTTTTGGGTTTCACTCACTCACTTCTTCCGACACAGGTGGAC  
E L E A D S F E L N N L S D L S N D L Q  
1860  
CAGTTGACTGCTTCTGTTAGTTAGTGTACCACTGCTTACAGGCGCGGATGAGAGAAC  
E V S S A G I L A S T V T D A G Y E C G  
1920  
GAGCGGCTGCTGCTACGTTGCGCGTACAGCGGCTTCACTGAGCGCGGATGAGAGAAC  
E R L V W A L A M D A L S Q C R E Q A Y  
1980  
CAGTAACGGAACGGCTGCTTATTAACAACTGCACTTGTACGGGAGTCTGTCCCT  
D N L K R V M D I N N V N L M G S S L S  
2040  
GAATCGGACCGGCGCGGCTTGGGAGACCTTAAAGTAGCTCTATAACAGCGCACTGCTC  
K C Q S A R S G E P I G D L Y K D T L L  
2100  
CGGAGCGGCTTACCACTCTTTCTTACTGCGGCTTCTGTAACACCGCTGAGTGAAGTT  
G Q A L P S F A I V C L G N N A A S Q L  
2160  
GTTCAAGAGTGGGAGTAGCGCGGCAAGGAGGTTGGTTGCTATTACTTCTTATCGAAC  
L N E G D D G P E E V W R N I I J Y S P  
2220  
GACTACTTATGCTTCTGCGGCTTGGGAGGAGGCTTACTTGAAGTGGCGGCGGAGCACTC  
Q H I L L P C G E E A M F E A A A T L  
2280  
GAGACCTGCGACCTTCTTTATGGGTCATTTCTCGGAAGGCTCTCGGTTGTTACAGTGC  
E P C Y S S I C V Y L A K G S A V I D R  
2340  
GTTACGCACTGACCAAGAGGTTTCTCTATAG  
L A Y M T E E L P T D  
2374





1 MVINEIFITG LLLGASLLLS IGPQNVLVIK QGIKREGLIA VLLVCLISDV  
TMH1  
51 ELFIAGTLGV DLLSNAAPIV LDIMRWGGIA YLLWFVAVMAA KQAMTNKVEA  
TMH2  
101 PQIIETEPT VPDDTPLGGS AVATDTRNRV RVEVSVDKQR VVVKPEMLMAI  
TMH3  
151 VLTLNPNAY LDAFVFIGV GAQYGDTRW IFAAGAFPAAS LIWFPLVGFG  
TMH4  
201 AAALSRLSS PKVWRWINVV VAVVMTALAI KLMLMG  
TMH5  
TMH6

Table 3

PROPER/PA110218-07.CLM-771099

- 15 -

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

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- 16 -

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Process for the microbial production of amino acids wherein a bacterial microorganism is modified such that the activity of the export carrier which is specific for the corresponding amino acid and which is encoded by a single export gene is increased and/or such that the expression of the single export gene specific for the corresponding amino acid of a bacterial microorganism producing the respective amino acid is increased.

2. Process according to claim 1, characterized in that the endogenous export carrier activity of the microorganism is increased.

3. Process according to claim 2, characterized in that by mutation of the endogenous export gene a carrier with higher export activity is generated.

4. Process according to one of the claims 1 to 3, characterized in that the gene expression of the export carrier is increased by increasing the number of gene copies.

5. Process according to claim 4, characterized in that to increase the number of copies the export gene is installed in a gene construct.

6. Process according to claim 5, characterized in that the export gene is installed in a vector with a low number of copies.

7. Process according to claim 5 or 6, characterized in that the export gene is installed in a gene construct which includes regulatory gene sequences assigned to the export gene.

8. Process according to claim 7, characterized in that the regulatory gene sequence includes a nucleotide sequence coding for the amino acid sequence given in table 1 and the allele variations thereof.

9. Process according to claim 9, characterized in that the



P10PERVPAUS210-97/CLM-7/12/99

- 17 -

regulatory gene sequence includes a nucleotide sequence of nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

10. Process according to one of the claims 5 to 9, characterized in that a microorganism producing the respective amino acid is transformed with the gene construct including the export gene.

11. Process according to claim 10, characterized in that a microorganism of the type *Corynebacterium* is transformed with the gene construct including the export gene.

12. Process according to claim 10 or 11, characterized in that for the transformation a microorganism is utilized in which the enzymes which participate in the synthesis of the corresponding amino acids are deregulated.

13. Process according to one of the claims 10 to 12, characterized in that for the transformation a microorganism is utilized which contains an increased part of the central metabolism metabolites.

14. Process according to one of claims 4 to 13, characterized in that the export gene is isolated from a microorganism strain of the type *Corynebacterium*.

15. Process according to one of the preceding claims, characterized in that the export gene sequence is identified by comparison with the sequence of an already known export gene.

16. Process according to claim 15, characterized in that that the amino acid sequence derived from the export gene sequence to be identified is compared with the amino acid sequence given in table 3 or the allele variation thereof.

17. Process according to one of the preceding claims, characterized in that the export gene expression is increased by amplifying the transcription signals.

18. Process according to one of the preceding claims, characterized in that as export gene, a gene with a nucleotide



PROPERVFAU2197.CLM - 1/10/99

- 18 -

sequence coding for the amino acid sequence given in table 3 and the allele variations thereof is utilized.

19. Process according to claim 18, characterized in that as export gene a gene with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 or a DNA sequence with essentially the same effects is utilized.

20. Process according to one of the preceding claims for the manufacture of L-lysine.

21. A single isolated or modified bacterial export gene coding for an amino acid export carrier.

22. Export gene according to claim 21 with a nucleotide sequence coding for an amino sequence given in table 3 or the allele variation thereof.

23. Export gene according to claim 22 with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 or a DNA sequence with essentially the same effects.

24. Export gene according to one of the claims 21 to 23 with regulatory gene sequences assigned thereto.

25. Export gene according to claim 24, characterized in that the regulating gene sequence includes a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

26. Export gene according to claim 25, characterized in that the regulating gene sequence includes a nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

27. An isolated or modified regulator gene suitable for the regulation of an export gene coding for an amino acid and export carrier, including a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

28. Regulator gene according to claim 27 with the nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

29. Gene structure containing an export gene according to



P:070517PA1121877 CLM - 14/100

- 19 -

one of claims 21 to 24.

30. Gene structure including a regulatory gene sequence according to claim 27 or 28.

31. Vector including an export gene according to one of claims 21 to 26 or a gene structure according to claim 29.

32. Vector according to claim 31 with a low number of copies.

33. Vector including a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

34. Transformed cell including, in a replicable form, an export gene according to one of the claims 21 to 26 or a gene structure according to claim 29.

35. Transformed cell according to claim 34 including a vector according to claim 31 or 32.

36. Transformed cell according to claim 34 or 35, characterized in that it belongs to the type *Corynebacterium*.

37. Transformed cell according to one of claims 34 to 36, characterized in that in this cell the enzymes of the amino acid, which participate in the synthesis, are deregulated and wherein the amino acid is removed from the cell by way of the export carrier for which the export gene, which was transferred into the transformed cell, codes.

38. Transformed cell according to one of claims 34 to 37, characterized in that the cell includes an increased proportion of central metabolism metabolites.

39. Transformed cell including, in replicable form, a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

40. Transformed cell according to claim 39, including a vector according to claim 33.

41. An isolated or modified bacterial membrane protein specific for the export of amino acids comprising 6 transmembrane helices.

42. Membrane protein according to claim 41, including the





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- 20 -

amino acid sequence given in table 3 wherein table 3 is part of this claim.

43. Use of a single isolated or modified bacterial export gene encoding an amino acid export carrier for increasing the amino acid production of micro-organisms.

44. Use according to claim 43, characterized in that a mutated export gene, which codes for an enzyme with increased export carrier activity is utilized.

45. Use according to claim 43 or 44, characterized in that the amino acid producing microorganism is transformed with a gene construct which includes an export gene.

46. Use according to claim 45, characterized in that the gene construct additionally carries regulatory gene sequences.

47. Use according to one of the claims 43 to 46, characterized in that an export gene from *Corynebacterium* is utilized.

48. Use according to one of claims 43 to 47, characterized in that *Corynebacterium* is used as amino acid producing microorganism.

49. A process according to any one of claims 1 to 20 or an export gene according to any one of claims 21 to 26 or a regulator gene according to any one of claims 27 and 28 or a gene structure according to claims 29 or 30 or a vector according to any one of claims 31 to 33 or a transformed cell according to any one of claims 34 to 40 or a membrane protein according to any one of claims 41 or 42 or a use according to any one of claims 43 to 48 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this 25th day of JULY, 2000

Forschungszentrum Jülich GmbH

DAVIES COLLISON CAVE

Patent Attorneys for the Applicant



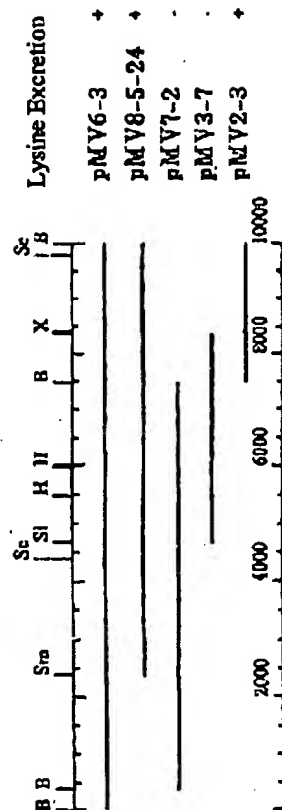


Figure 1

CgLyse 1 MVIMPIFITGLLLCASLLLSIGPONVIVIKOGIKRDELIAVLLVCLTSOV 50  
 EcYga 1 .....MILPLGPONAFVHNQGIARQYHIMIALCAISDL 34  
  
 CgLyse 51 FLFIAGTIGVDLLSNAAPIVLDIMRWGGIAYLLWFAVDAJQDMYKVEA 100  
 EcYga 35 VLICAGYFGGSALLNQSPWLLALVTWGGVAFLLWYGGAFKAMSSNIS 83  
  
 CgLyse 101 POIIEETEPTVPDPTPLGGSAVATDTRNRVRVEVSVDKORVWVKPMLHAY 150  
 EcYga 84 .....LASAEVHKQURWK .....YIATHLAV 104  
  
 CgLyse 151 VLINLNPAYLDAFVFIGGVRAOYGDTCWTIFACAPASLIWFLVGFQ 200  
 EcYga 105 ..TWLNPHVYLDTFVVLGSLGGQLDVEPKRWFAIGTISASFVWFFGLAL 152  
  
 CgLyse 201 AAALSRLSSPKVWRWYNVAVVHTALAIKLMNG ..... 236  
 EcYga 153 AAWLAPRLRTAKAQRTYNLVVCCVWFIALQLAKDGIAHAQALFS 197

Figure 2

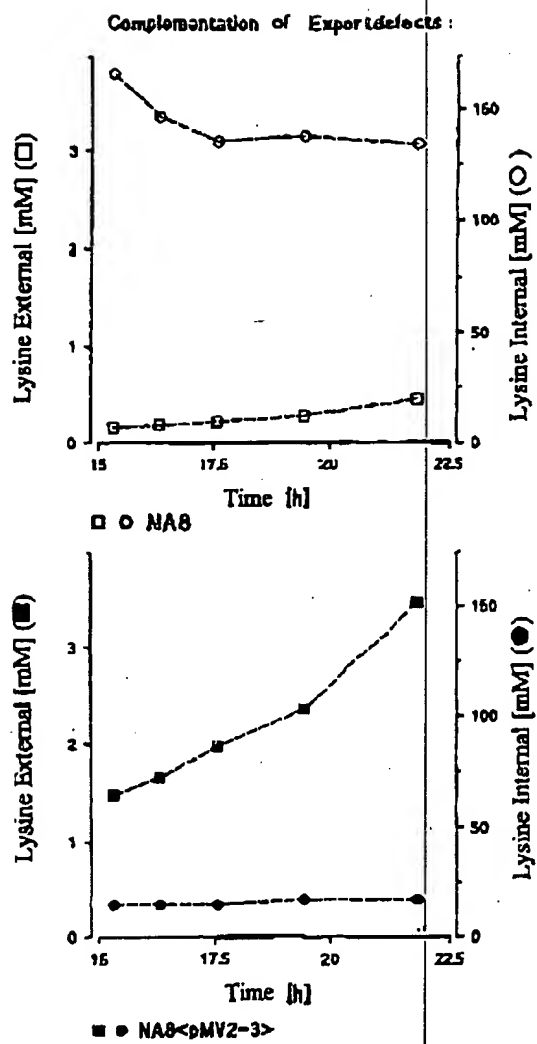


Figure 3

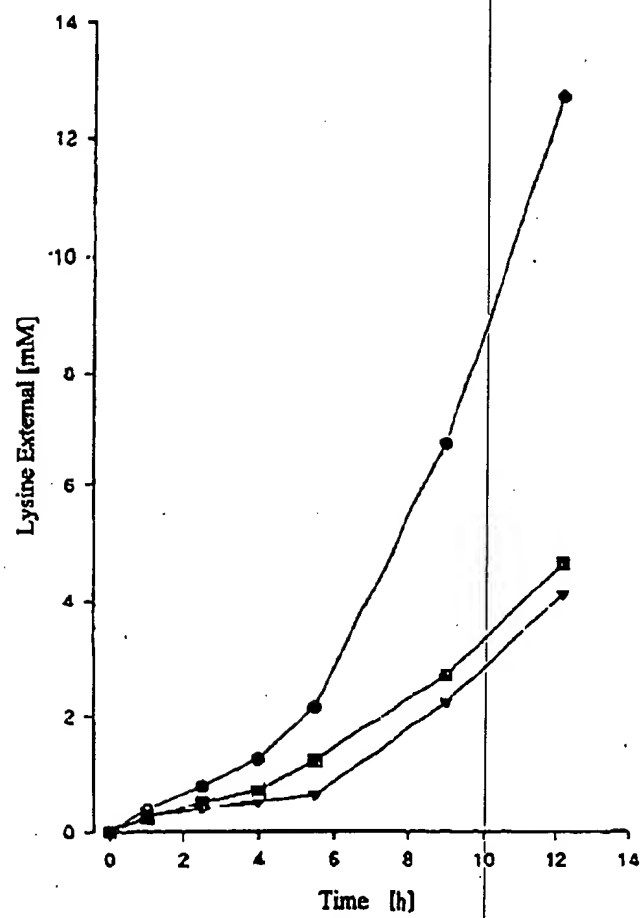


Figure 4